Parasites of African penguins: diversity, ecology and effect on hosts

by

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Declaration

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The content in each data chapter (Chapters 2-5) is written as an original paper to be published in a peer-reviewed journal. There is therefore some overlap of the information between the chapters.

This research project was approved by the Animal Ethics Committee of the University of Stellenbosch (reference number SU-ACUD15-00114) and permits were obtained from the Division of Environmental Affairs (RES2016/95 and RES2017/02), the Threatened or Protected Species (TOPS) of the Biodiversity Act (07962), CapeNature (AAA007-00191-0056) and South African National Parks (CRC/2016-2017/038--2015/V1).

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Abstract

The African penguin (*Spheniscus demersus*) is a critically endangered seabird species endemic to southern Africa. Recent reports of soft ticks on penguins raised concerns that parasites may pose a risk to the species' conservation. To date, it is uncertain if parasite populations are similar across colonies and what factors drive parasite infestations. It is further uncertain if current parasite burdens affect penguin health. The aims of the study were to: (1) record the parasite diversity associated with African penguins and their nests across penguin colonies, and determine the factors that shape parasite infestations; (2) document clinical parameters for wild African penguins and establish the relationship between parasite infestations and penguin health across colonies; (3) at a local scale, record the relationship between nest characteristics and nest ectoparasites and determine the potential impact on the health of African penguins at the Stony Point colony; and (4) ascertain the efficiency of a modified Berlese funnel system, with naphthalene as repellent, as a quantitative method for the extraction of nest ectoparasites. Adult penguins (210), chicks (583) and nests (628) were sampled across five colonies along the south-western coast of South Africa in the autumn/winter season in 2016 and 2017, and also in spring 2016 at the Stony Point colony. Ectoparasites were recorded on all penguins and in nests. Helminths were recorded from chick faecal material. Blood samples were screened for haemoparasites and health parameters recorded. Penguin age and morphometric measurements (chicks) were recorded. Across colony data included nest density and weather conditions, while nest characteristic and microclimatic conditions in nests were recorded at Stony point. Ectoparasites (*Parapsyllus humboldti*, *Echidnophaga gallinacea* and *Ornithodoros capensis* s. s.), haemoparasites (Piroplasmorida/Haemospororida and Spirochaetales) and helminth parasites (*Cardiocephaloides* spp., *Renicola* spp., *Contracaecum* spp. and *Cyathostoma* spp.) were recorded from penguins, while ticks and fleas were recorded from their nests. At a regional scale, parasite infestations were higher in chicks than adult penguins; mainland colonies recorded more on-host and in-nest ectoparasites, Piroplasmorida/Haemospororida and *Cardiocephaloides* spp. than islands. Nest ticks, Piroplasmorida/Haemospororida and *Cardiocephaloides* spp. infecting penguins were positively associated with total nest density, while total nest ectoparasites increased with active nest density. Clinical health parameters of wild African penguins varied among colonies and several parameters were adversely affected by ecto- and haemoparasite species richness, but positively related to helminth species richness. At Stony Point, tick abundance in addition to ecto- and haemoparasite richness adversely affected haematocrit values. Chick body condition was significantly lower in spring compared to autumn/winter. At a local scale, tick and flea

infestations were higher in artificial nests, nests close to the coastline, warmer and drier nests. Flea burdens were higher in nests occupied by a penguin. Conditions associated with artificial nests were not significantly related to penguin health parameters. Climatic conditions associated with spring were negatively related to on-host and in-nest parasite infections and clinical health parameters. The modified Berlese funnel consistently underestimated the abundance and prevalence of all ectoparasites in nest samples and particularly more so for the abundance of flea larvae. To conclude, although parasites are widely associated with African penguins it seems that at present penguins are able to withstand current infestation levels at most colonies. Regional differences in parasite infestation patterns may be driven by the eastward migration of prey fish, which in the case of Stony Point is intensified by the ability of ticks and fleas to take advantage of conditions associated with artificial nests.

Opsomming

Die Afrika-pikkewyn (*Spheniscus demersus*) is 'n krities bedreigde seevoëlspesie wat endemies is in Suider-Afrika. Onlangse verslae van sagte-bosluise op pikkewyne wek kommer oor die risiko wat parasiete vir die spesie se bewaring inhou. Tot op datum is dit onseker of parasietbevolkings in alle kolonies soortgelyk is en watter faktore parasietbesmettings bevorder. Dit is verder onseker of huidige parasietladings die gesondheid van pikkewyn beïnvloed. Die doelstellings van die studie was: (1) om die parasietdiversiteit wat met Afrika-pikkewyne en hul neste in verskeie kolonies geassosieer word aan te teken, en die faktore wat parasietbesmetting veroorsaak te bepaal; (2) die dokumentasie van kliniese waardes vir wilde Afrika-pikkewyne en om die verband tussen parasietbesmettings en pikkewyngesondheid oor kolonies te bepaal; (3) om op 'n plaaslike skaal die verband tussen nes-kenmerke en nes-ektoparasiete waar te neem en die potensiële impak op die gesondheid van Afrika-pikkewyne by die Stony Point-kolonie te bepaal; en (4) om die doeltreffendheid van 'n aangepaste Berlese tregterstelsel, met naftaleen as afweermiddel, as 'n kwantitatiewe metode vir die onttrekking van nes-ektoparasiete vas te stel. Volwasse pikkewyne (210), kuikens (583) en neste (628) is in die herfs / winterseisoen in 2016 en 2017 oor vyf kolonies langs die suidwestelike kus van Suid-Afrika bestudeer en ook in die lente van 2016 by die Stony Point-kolonie. Ektoparasiete is op alle pikkewyne en in hul neste aangeteken, bloedmonsters was bestudeer vir hemoparasiete en klinies-gesondheidswaardes is aangeteken. Die ouderdomsgroep van elke pikkewyn was ook bepaal. Verder, monsters wat by kuikens geneem is sluit in mismonsters om die teenwoordigheid van helminth-parasiete te noteer en morfometriese metings om die kondisie van die diere te bepaal. Oor kolonie-data het nes-digtheid en weerstoestande ingesluit, terwyl nes-kenmerke en mikroklimaat toestande in neste op Stony Point aangeteken is. Ectoparasiete (*Parapsyllus humboldti*, *Echidnophaga gallinacea* en *Ornithodoros capensis* s. s.), haemoparasiete (Piroplasmorida/Haemospororida en Spirochaetales) en helminth parasiete (*Cardiocephaloides* spp., *Renicola* spp., *Contracaecum* spp. en *Cyathostoma* spp.) was op die pikkewyne aangeteken, terwyl bosluise en vlooie in hul neste aangeteken is. Op 'n streekskaal was parasietbesmettings hoër op kuikens as volwasse pikkewyne; vasteland kolonies het meer ektoparasiete op pikkewyne en in hul nests gehad as ook meer Piroplasmorida/Haemospororida en *Cardiocephaloides* spp. in vergelyking met eilande. Bosluise in neste en Piroplasmorida/Haemospororida en *Cardiocephaloides* spp. besmette pikkewyne was positief geassosieer met totale nes-digtheid, terwyl die totale ektoparasietlading in neste met die digtheid van aktiewe-neste toegeneem het. Kliniese-gesondheidswaardes van wilde Afrika-pikkewyne het tussen kolonies verskil en verskeie van die waardes is nadelig beïnvloed deur

ekto- en hemoparasiet-spesiesrykheid, maar was positief verwant aan helminth-spesiesrykheid. By Stony Point, het bosluisladings in kombinasie met ekto- en hemoparasiet-spesiesrykheid die hematokrit-waardes nadelig beïnvloed. Die liggaamskondisie van kuikens was aansienlik laer in die lente in vergelyking met herfs / winter. Op 'n plaaslike skaal was bosluis- en vlooibesmettings hoër in kunsmatige neste, neste naby die kuslyn, warmer en droër neste. Vlooibesmettings was hoër in neste waar pikkewyne teenwoordig was. Toestande wat geassosieer was met kunsmatige-neste het nie ’n beduidend invloed op die gesondheidwaardes van pikkewyn gehad nie. Klimaatstoestande wat geassosieer was met lente was negatief verwant aan parasietinfeksies op pikkewyne en in hulle neste en kliniese-gesondheidwaardes. Die gemodifiseerde Berlese tregterstelsel het die lading en voorkoms van alle ektoparasiete en veral die lading van vlooi larwes in nes-monsters onderskat. Ter afsluiting, alhoewel parasiete wyd verspreid op Afrika-pikkewyne voorkom, blyk dit dat huidige infestasievlakke nie die pikkewyne negatief beïnvloed nie. Streeksverskille in parasietbesmettingspatrone word moontlik aangedryf deur die oostelike migrasie van prooivis, wat in die geval van Stony Point vererger word deur die vermoë van bosluise en vlooie om voordeel te trek uit toestande wat met kunsmatige-neste verband hou.

Dedication

This thesis is dedicated to my Professor and friend, Dr Roberto Schlatter Vollmann. For inspiring me to listen to what nature tells us, observe it with respect and devote oneself to it.

Esta tesis está dedicada a mi profesor y amigo, Dr. Roberto Schlatter Vollmann. Por inspirarme a escuchar lo que la naturaleza nos dice, observarla con respeto y dedicarle una vocación de amor. ¡Gracias profe!

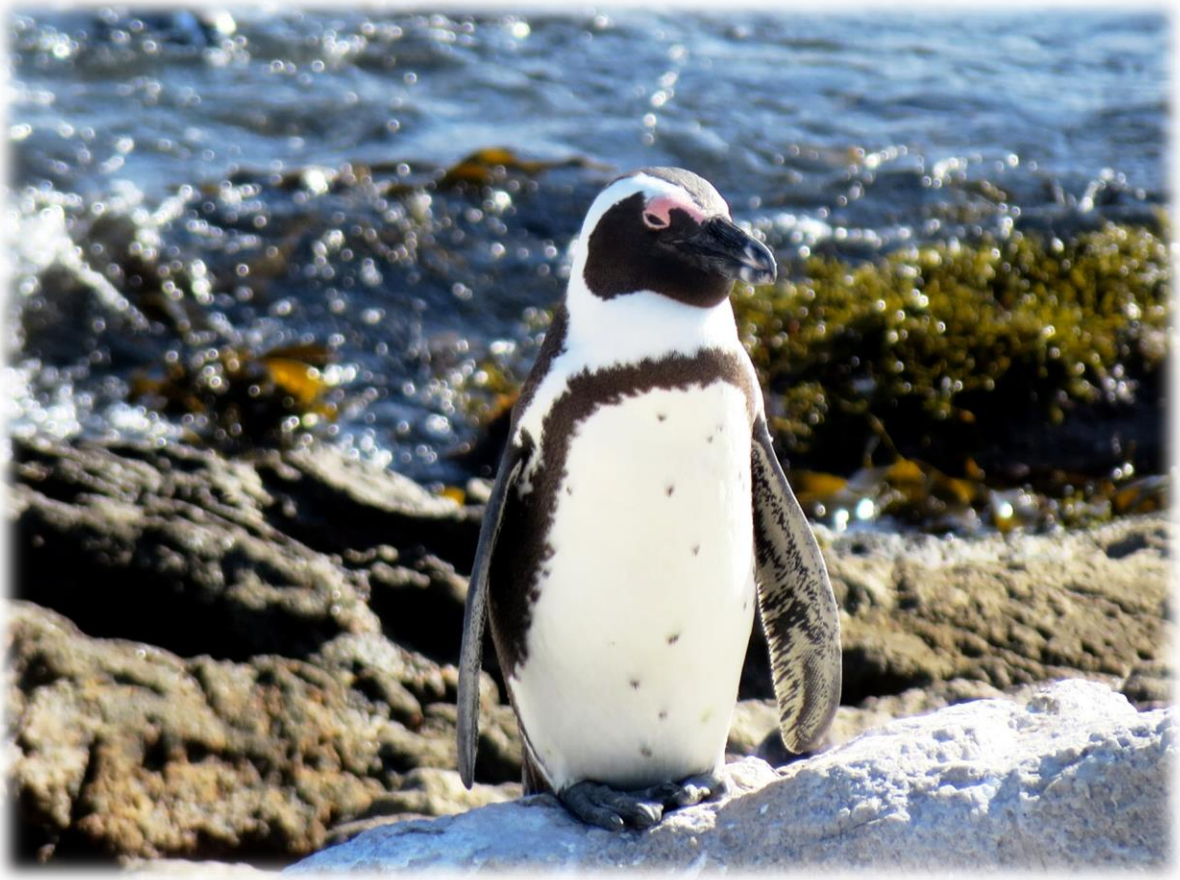

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Figure S2.3. Cross-colony differences between mean prevalence of Piroplasmids/Haemospororida and (A) total nest density (average/m^2): Dassen Island 0.02, Robben Island 0.05, Dyer Island 0.08, Simon's Town 0.22, Stony Point 0.28; (B) active nest density (average/m^2): Robben Island 0.007, Dassen Island 0.012, Dyer Island 0.06, Stony Point 0.13, Simon's Town 0.14; (C) annual mean temperature (°C): Dassen Island 15.6, Stony Point 16.3, Dyer Island 16.4, Simon's Town 16.5, Robben Island 16.6; (D) annual precipitation (mm): Dassen Island 381, Dyer Island 521, Stony Point 611, Simon's Town 693, Robben Island 724.

Figure S2.4. Cross-colony differences between mean prevalence of *Cardiocephaloides* spp. and (A) total nest density (average/m^2): Dassen Island 0.02, Robben Island 0.05, Dyer Island 0.08, Simon's Town 0.22, Stony Point 0.28; (B) active nest density (average/m^2): Robben Island 0.007, Dassen Island 0.012, Dyer Island 0.06, Stony Point 0.13, Simon's Town 0.14; (C) annual mean temperature (°C): Dassen Island 15.6, Stony Point 16.3, Dyer Island 16.4, Simon's Town 16.5, Robben Island 16.6; (D) annual precipitation (mm): Dassen Island 381, Dyer Island 521, Stony Point 611, Simon's Town 693, Robben Island 724.

Figure S2.5. Cross-colony differences between mean abundance of total nest ectoparasites and (A) total nest density (average/m^2): Dassen Island 0.02, Robben Island 0.05, Dyer Island 0.08, Simon's Town 0.22, Stony Point 0.28; (B) active nest density (average/m^2): Robben Island 0.007, Dassen Island 0.012, Dyer Island 0.06, Stony Point 0.13, Simon's Town 0.14; (C) annual mean temperature (°C): Dassen Island 15.6, Stony Point 16.3, Dyer Island 16.4, Simon's Town 16.5, Robben Island 16.6; (D) annual precipitation (mm): Dassen Island 381, Dyer Island 521, Stony Point 611, Simon's Town 693, Robben Island 724.

Figure S2.6. Cross-colony differences between mean abundance of nest fleas and (A) total nest density (average/m^2): Dassen Island 0.02, Robben Island 0.05, Dyer Island 0.08, Simon's Town 0.22, Stony Point 0.28; (B) active nest density (average/m^2): Robben Island 0.007, Dassen Island 0.012, Dyer Island 0.06, Stony Point 0.13, Simon's Town 0.14; (C) annual mean temperature (°C): Dassen Island 15.6, Stony Point 16.3, Dyer Island 16.4, Simon's Town 16.5, Robben Island 16.6; (D) annual precipitation (mm): Dassen Island 381, Dyer Island 521, Stony Point 611, Simon's Town 693, Robben Island 724.

Chapter 3

Table S3.1. Model selection based on Akaike Information Criterion (AIC) across colonies. Numbers in brackets represent the different scenarios derived from the full model (assessed in the study) to reach the best model (in bold). Significant differences between the full and the best model (p-value) according to Chi-square test. Independent variables using parasite abundance: fleas, ticks, lice, colony (Stony Point, Simon's Town, Dassen-, Dyer- and Robben Island), year (2016 and 2017), age (adult and chick). Independent variables using parasite richness: ectoparasites (fleas, ticks and lice), haemoparasites (Piroplasmorida/Haemospororida and Spirochaetales), helminth parasites (*Cardiocephaloides* spp., *Renicola* spp., *Contracaecum* spp. and *Cyathostoma* spp.), colony (Stony Point, Simon's Town, Dassen-, Dyer- and Robben Island), year (2016 and 2017) and age (adult and chick).

Table S3.2. Model selection based on Akaike Information Criterion (AIC) at Stony Point. Numbers in brackets represent the different scenarios derived from the full model (assessed in the study) to reach the best model (in bold). Significant differences between the full and the best model (p-value) according to Chi-square test. Independent variables using parasite abundance: fleas, ticks, lice, season (autumn/winter and spring season), age (adult and chick) and year (2016 and 2017). Independent variables using parasite richness: ectoparasites (fleas, ticks and lice), haemoparasites (Piroplasmorida/Haemospororida and Spirochaetales), helminth parasites (*Cardiocephaloides* spp., *Renicola* spp., *Contracaecum* spp. and *Cyathostoma* spp.), year (2016 and 2017), age (adult and chick) and season (autumn/winter and spring season).

Chapter 4

Table S4.1. Nest characteristics assessed for African penguins at the Stony Point penguin colony along the west coast of South Africa. The mean value (±SE) of and proportion (%) per nest type and sampling season is presented. Sampling seasons: autumn/winter 2016 (SP1); spring 2016 (SP2); and autumn/winter 2017 (SP3).

Table S4.2. Model selection based on Akaike Information Criterion (AIC). Numbers in brackets represent the different scenarios derived from the full model (assessed in the study) to reach the best model (in bold). Significant differences between the full and the best model (p-value) according to Chi-square test. Independent variables: nest type (artificial, natural covered and natural open nests), nest occupancy (active or inactive), nest age (nests established within a year, nests established more than one and less than three years ago, and nests established more than three years ago), distance to the south-east coast (m), distance to the west coast (m),

nest position (windward or leeward) nest opening (cm) and SP (sampling seasons: autumn/winter 2016, spring 2016 and autumn/winter 2017).

Table S4.3. Model selection based on Akaike Information Criterion (AIC). Numbers in brackets represent the different scenarios derived from the full model (assessed in the study) to reach the best model (in bold). Significant differences between the full and the best model (p-value) according to Chi-square test. Independent variables: nest type (artificial, natural covered and natural open nests), nest occupancy (active or inactive), nest age (nests established within a year, nests established more than one and less than three years ago, and nests established more than three years ago), distance to the south-east coast (m), distance to the west coast (m), nest position (windward or leeward) nest opening (cm), temperature mean (°C) + temperature SD (°C) + moisture of nest soil (%) + moisture of nest material (%) and SP (sampling seasons: autumn/winter 2016, spring 2016 and autumn/winter 2017).

Table S4.4. Model selection based on Akaike Information Criterion (AIC). Numbers in brackets represent the different scenarios derived from the full model (assessed in the study) to reach the best model (in bold). Significant differences between the full and the best model (p-value) according to Chi-square test. Independent variables: nest type (artificial, natural covered and natural open nests), distance to the south-east coast (m), distance to the west coast (m), penguin ectoparasites (abundance), temperature mean (°C) + moisture of nest soil (%) + moisture of nest material (%), SP (sampling seasons: autumn/winter 2016, spring 2016 and autumn/winter 2017) and penguin age (adult penguins and chicks).

Chapter 1

General Introduction

1. Parasites in the ecosystem

1.1 Importance of parasites in the ecosystem

Parasites are broadly recognised as organisms that make use of another organism (host) to obtain nutrients and complete their life cycle and, in doing so, exert a degree of damage to the host (Price, 1980; Thomas *et al.* 2000). Parasites represent about half of the diversity of life on the planet (Price, 1980) and include species well adapted to inhabit different host species and their environments (Poulin and Morand, 2000; Fryderyk and Izdebska, 2009). Parasites comprise organisms from several phyla, including macroparasites such as ticks (Ixodida), mites (Acarina), fleas (Siphonaptera), lice (Phthiraptera), flies (Diptera), helminths (Platyhelminthes, Nematoda and Acanthocephala) (Poulin, 1997; Poulin and Morand, 2000) and microparasites such as bacteria, virus, protozoan and fungi (Anderson and May, 1981). Certain parasites live permanently in or on the host and are dependent on the host to complete their life cycle (e.g. lice and protozoan haemoparasites) (Rothschild and Clay, 1952; Borgsteede, 1996), while others spend their life cycle between the host body and the external environment (e.g. ticks, fleas and gastrointestinal nematodes) (Sonenshine, 1993; Bitam *et al.* 2010; Benesh *et al.* 2014).

Parasites are important components of biodiversity and they influence several ecological and evolutionary processes (Clayton *et al.* 1999; Gómez and Nichols, 2013). Important functions comprise the control of host populations by directly reducing host survival (Lehmann, 1993) or making them more susceptible to predators (Parker *et al.* 2003). Parasite diversity improves ecosystem functioning because they stimulate host diversification by influencing host reproduction and phenotypic characteristics (Gómez and Nichols, 2013). Their capacity for infecting several hosts and being transmitted through different trophic interactions makes parasites inexorably linked to food webs (Marcogliese and Cone, 1997). Therefore, their participation in trophic chains contributes to the flow of energy and the connectivity between the different trophic levels (Hudson *et al.* 2006). Other important functions of parasites include the regulation of the presence of other harmful parasite taxa (Thomas *et al.* 2000), and their role as bioindicators in polluted environments or anthropogenic habitats (Sures, 2003; Palm and Rückert, 2009).

1.2 Parasite effects and diseases

Despite their benefits on the ecosystem, parasites are mostly recognized by the detrimental effects they exert on their hosts (Gómez and Nichols, 2013). Parasites can produce a variety of unfavourable conditions in their hosts, which ranges from moderate skin irritation to threatening their survival (Lehmann, 1993; Sonenshine, 1993). Parasites can directly affect the host through

their physical presence or their feeding behaviour. Direct effects include irritation and damage of the integument (e.g. skin, feathers) by ectoparasites (e.g. ticks, fleas, lice, mites) that shelter themselves, reproduce and feed on skin debris and blood of their host (Proctor and Owens, 2000; Johnson and Clayton, 2003). High loads of ectoparasites cause stress, weakness and irritation (Gauthier-Clerc *et al.* 1998). This induces hosts to spend long periods preening and grooming themselves or each other (i.e. allopreening and allogrooming) rather than spending that time and energy on feeding, reproducing or taking care of their young ones (Brooke, 1985; Lehmann, 1993). Parasites can reduce offspring growth (Duffy, 1983) as well as negatively affect host reproduction (e.g. reduced fecundity and cause abortion and sterility) (Thomas *et al.* 2000; Gómez and Nichols, 2013). Furthermore, high ectoparasite infestations can cause adult birds to abandon eggs and chicks (King *et al.* 1977; Duffy, 1983). Blood sucking arthropods, endoparasites and haemoparasites can cause anemia (i.e. reduce the number or proportion of red blood cells) as a result of the blood lost or destruction of red cells experienced by the hosts (Sonenshine, 1993; Campbell and Ellis, 2007). The multiple injuries caused by parasites represent an energetic cost to the hosts, which generates an increase in metabolic expenditure to compensate the damage, thus altering host fitness (Møller *et al.* 1994). This effect is reflected in the loss of body condition and body mass in affected hosts (Hughes and Page, 2007; Norte *et al.* 2013). Endoparasites can affect the structure and function of internal organs. For example, helminth parasites (such as *Cardiocephaloides* spp.) that damage the gastrointestinal mucosa of their final (definitive) host can cause diarrhoea, malabsorption of nutrients with subsequent emaciation (Hansen and Perry, 1994). Further, parasites located in the respiratory tract (such as *Cyathostoma* spp. and *Trichomonas* spp. in avian hosts) can cause obstruction of airways, coughing, pneumonia and haemorrhage (Lavoie *et al.* 1999). Although parasites rarely kill their host, mortality due to an increase in abundance and richness of pathogenic parasite species can occur (Borgsteede, 1996; Johnson and Hoverman, 2012).

Parasites can also indirectly affect their hosts by affecting the host's immune system and by transmitting pathogenic agents (Jongejan and Uilenberg, 2004). Parasites can cause an overreacted host immune response with a resultant harmful effect for the same animal, and make the host more susceptible to environmental threats (e.g. lack of food, adverse climatic conditions or presence of predators) that may compromise its survival (Borgsteede, 1996; Thomas *et al.* 2000; Sorci, 2013). The presence of parasites (mostly arthropods) that serve as vectors of pathogens can also contribute to host mortality (Nuttall, 1984). For instance, about 10% of the existing soft and hard tick species are vectors of a great variety of pathogens, including rickettsia, protozoa and viruses (Sonenshine, 1993; Jongejan and Uilenberg, 2004). Fleas carry important bacterial, viral and rickettsial diseases such as the plague (*Yersinia pestis*)

in rodents and humans (Krasnov, 2008; Bitam *et al.* 2010). Lice can transmit bacteria, fungi and helminths (Barlett, 1993; Johnson and Clayton, 2003) and mites are vectors of rickettsia, viruses and protozoa (Proctor and Owens, 2000). Mosquitoes are known for carrying viruses, bacteria, protozoa and filarial nematodes (Boyd, 1951; Daszak *et al.* 2000). Several studies have recorded fatal consequences for animal hosts, including the multiple deaths of red foxes (*Vulpes vulpes*) after the introduction and rapid spread of mites (*Sarcoptes scabiei*) in Sweden, Finland and Norway (Mörner and Christensson, 1984); the acute mortality of domestic bovids during the early 1900`s following the introduction of a protozoan parasite (*Theileria parva*) (East Coast fever) in South Africa, which is transmitted by Ixodid ticks (*Rhipicephalus appendiculatus*) (Yusufmia *et al.* 2010); and the mass extinction of endemic birds in Hawaii due to the avian malaria parasite (*Plasmodium relictum*) transmitted by mosquitoes (*Culex quinquefasciatus*) (Warner, 1968).

The direct and indirect effects of parasites associated with birds are of special interest in the study of infectious diseases (Friend and Franson, 1999). The numerous extant species of birds (currently ca. 18,000 bird species in the planet; Barrowclough *et al.* 2016), the variety of environments they occupy (e.g. urban and rural, marine and terrestrial and nesting on the ground or forest canopy; Schreiber and Burger, 2001; Delgado and French, 2012; Lutz *et al.* 2015), the long distance migration they attain during seasonal movements (Ricklefs *et al.* 2017) and gregarious habits they can exhibit (such as seabirds; Schreiber and Burger, 2001) promote host-parasite interactions that can facilitate parasitic transmission and dispersion, and extend the effect of parasites to hosts in different geographic areas (Tella, 2002; Lion *et al.* 2006)

2. Factors affecting host-parasite dynamics

Parasite communities comprise multiple species that co-exist in the host and the environment (Bordes and Morand, 2009; Johnson and Hoverman, 2012). Given their potential to affect their host it is important to know the factors that affect parasite diversity, their abundance and distribution, in order to understand and foresee the likelihood of parasitic colonization and development (Poulin and Morand, 2000). For example, when studying the effect of changes in host (e.g. host abundance and density) or environmental factors (e.g. vegetation structure and climate) on infection and transmission of different parasites, the individual responses can be better understood when considering parasite life history (e.g. parasite interaction and mode of transmission) (Vicente *et al.* 2007).

2.1 Parasite life history

The life history characteristics of parasites not only influence their local abundance and prevalence but also play an important governing role in their regional distribution (Mendes *et al.* 2005; Barrett *et al.* 2008). Parasites exhibit differences in the number of hosts, transmission mode and type of habitat they use to complete their life cycles (Barrett *et al.* 2008). Some parasites live their entire lives on/in the host (i.e. permanent parasites), whereas other parasites are more facultative in the use of the hosts and spend part of their life cycle in the environment (Johnson and Clayton, 2003; Benesh *et al.* 2014). While off-host, parasites exhibit differences in habitat use. Some parasites (nidicolous parasites) are adapted to live under the climatic conditions offered in or near the host shelter (e.g. nests, burrows and caves) and others (non-nidicolous parasites) spend most of their life in the exposed environment (Sonenshine, 1993). Across the spectrum of host and habitat use, parasites may colonize only one host species (direct life cycle) to complete their life cycle, or multiple host species (indirect or complex life cycle) where parasites use intermediate host species before reaching the final host (Jongejan and Uilenberg, 2004; Benesh *et al.* 2014).

General life history traits of bird parasites include the nidicolous life style of Argasidae ticks, such as *Argas* spp., which are found in nests of passerines (Sonenshine, 1991), and *Ornithodoros* spp. that are usually found infesting seabird nests in temperate areas (Clifford *et al.* 1980; Dupraz *et al.* 2016). In the majority of soft ticks, each life stage (larva, nymph and adults) requires at least one blood meal on a vertebrate host prior to moulting (Sonenshine, 1991). Soft ticks spend little time feeding on their hosts, especially adults and nymphs that spend from a few minutes to an hour to complete a blood meal, while larva can take hours to days (Oliver, 1989; Vial, 2009). Consequently, soft ticks spend most of their time hiding in cracks, crevices or among the nest material in the nest of the host (Sonenshine, 1993). Argasid ticks are likely to move from one suitable host to another within the same area (Dupraz *et al.* 2016). However, they are also able to live for years without the presence of a host and a blood meal (Anderson and Magnarelli, 2008). Once fed, the female lays several small egg batches (5-500 per cycle) regardless of previous copulation and is able to produce up to five clutches over a lifetime (Vial, 2009). Argasid ticks exhibit several nymphal stages (Jongejan and Uilenberg, 2004) and can live for many years, reaching a lifespan of 25 years (Sonenshine, 1993). Transmission of soft ticks takes place in or near the nest occupied by the host (Sonenshine, 1991).

Some hard ticks (Ixodidae) are also nidicolous parasites of birds, such as *Ixodes uriae*, which infests seabird nests in the Antarctic and subantactic region (Brooke, 1985; Benoit *et al.* 2007; Gauthier-Clerc *et al.* 1998). All the life stages of hard ticks require a blood meal from a

suitable host (Oliver, 1989). Each life stage of *Ixodes* spp. ticks attach to a different host to engorge, then drops off and moult to the next life stage in the environment (e.g. bird`s nest) until they reach the adult stage (i.e. they complete a three-host life cycle) (Jongejan and Uilenberg, 2004). In particular, *I. uriae* uses a questing and waiting scheme to find their seabird hosts (Muzaffar and Jones, 2007). Blood-feeding time is longer when compared to Argasidae ticks (it takes several days) (Klompen *et al.* 1996). After feeding, females lay large batches of eggs (3000 or more) and then die (Oliver, 1989; Anderson and Magnarelli, 2008). There is only one nymphal instar (Klompen *et al.* 1996) and in general hard ticks have a shorter lifespan than Argasidae ticks (e.g. 1 year or less; Sonenshine, 1993). Transmission of nidicolous hard ticks also takes place in or near the hosts nest (Benoit *et al.* 2007). The only representative of Nutallidae ticks (*Nuttalliella namaqua*) is found in nests of lesser striped swallows (*Hirundo abyssinica*) in South Africa, Namibia and Tanzania (Oliver, 1989; Sonenshine, 1991). Although little is known about the life cycle of Nutallidae ticks, some morphological characteristics are similar to Argasidae and Ixodidae ticks (Sonenshine, 1991).

Mites are arachnids that display parasitic or predatory behaviour (Walter and Proctor, 2013). Some species of mites are also associated with bird nests, such as mites from the genera *Ornithonyssus* and *Dermanyssus* (order Mesostigmata) (Proctor and Owens, 2000; Roy and Chauve, 2010). The life stages of mites include egg, prelarva, larva, protonymph, deutonymph, tritonymph (not present in Mesostigmata) and adult (Proctor and Owens, 2000; Walter and Krantz, 2009). Nest dwelling parasitic mites colonize the integument where they feed on skin, feather's oil, feather pith, exuding tissue fluids and blood (Furman, 1959; Proctor, 2003). Prelarva is normally the nonfeeding stage. Many larva are also nonfeeding (e.g. Mesostigmata) (Walter and Krantz, 2009), while nymphal stages and adults are usually blood feeders (Moreno *et al.* 2009). Following a blood meal, female nest mites lay a small number of eggs (e.g. *Dermanyssus* spp. ca. 20 eggs and *Ornithonyssus* spp. ca. 2-5 eggs per clutch; Møller, 1990; Krantz, 2009), the life cycle is however short (e.g. 5-7 days in *Ornithonyssus* spp.). Therefore, mites can build up large populations rapidly, reaching thousands in bird nests during nesting of adult birds and the chick-rearing period (Møller, 1990; Moreno *et al.* 2009). Nest mites are transmitted by interactions and physical contact of birds, while they can also walk from one host to another especially when the hosts are grouped in dense communities (Proctor and Owens, 2000). Parasitic mites show different degrees of host specificity, i.e. some infect one host species (monoxeny) while others many host species (polyxeny). However, parasitic nest mites may prefer hosts based on the availability in the environment (nest) (Krantz, 2009).

Fleas are parasitic insects that undergo complete metamorphosis (Boyd, 1951). Some nidicolous fleas include the hen flea (*Ceratophyllus gallinae*) and the sticktight flea

(*Echidnophaga gallinacea*) (Merino and Potti, 1996; Boughton *et al.* 2006) found in nests of passerines, *Monopsyllus indages* from nests of woodpeckers (Kiefer *et al.* 2010) and fleas from the genus *Parapsyllus* found in seabird nests (Jordan, 1942). Only the adult stage feeds on the blood of the host, while the eggs, larvae and pupa remain in the host's nest and in a few species, on the host body (Bitam *et al.* 2010). Female fleas are able to lay a total of 300-500 eggs (Rothschild and Clay, 1952). After hatching larvae can undertake three instars depending on the availability of food (host skin or organic substrate in the host nest) and prevailing environmental conditions (Krasnov, 2008; Bitam *et al.* 2010). Environmental factors are also critical for the development of the other life stages of fleas, such as the water balance of pupae and the time of emergence of adults from the cocoons (Krasnov, 2008). Although fleas rarely exhibit preference for the same host species, fleas tend to parasitize related (taxonomically and ecologically) hosts. Nest fleas particularly tend to show more specificity for their hosts in nests (Bitam *et al.* 2010). During periods of host absence in the nest, fleas remain dormant as cocoons until a stimulus induces the adult emergence (e.g. rise of temperatures). Adult fleas are then able to jump onto birds from its own or other nests to obtain food (Bates, 1962, Tripet *et al.* 2002).

Lice are obligate permanent ectoparasites that spend their entire life cycle on the body of the host (Marshall, 1981). There are two taxonomic groups in lice: chewing lice (Mallophaga), that feed on feathers, dermal debris, secretions, blood and other microorganisms (Johnson and Clayton, 2003), and sucking lice (Anoplura) that feed on blood (Durden, 2001). Birds are parasitized by chewing lice (Amblycera, Ischnocera and Rhynchophthirina), many of which are host specific especially Ischnocera (Johnson and Clayton, 2003). Some chewing lice that parasitize bird species include *Pectinopygus* spp., which infest seabirds (Rivera-Parra *et al.* 2014), *Sturnidoecus* spp. found on starlings (Johnson and Clayton, 2003) and *Austrogoniodes* spp., which is a typical lice genus that parasitize penguins (Banks *et al.* 2006). Lice cannot survive more than a few days off the host because they depend on the temperature and humidity conditions offered by the host skin (Tompkins and Clayton, 1999). The life stages of lice include eggs, nymphs (three instars) and adults (Durden, 2001). The successful development of each life stage depends on the environmental conditions near the host skin (Nelson and Murray, 1971; Johnson and Clayton, 2003). Transmission of lice occurs by direct physical contact between individuals, and therefore the proximity (density) of hosts is an important factor that promotes lice infestations (Clayton and Tompkins, 1995; Rivera-Parra *et al.* 2014). Transmission is by direct contact between hosts (such as between parents and chicks or between copulating birds), and via vectors that transport lice from one host to another more distant (i.e. phoresis) (Clayton *et al.* 1992; Johnson and Clayton, 2003).

Certain groups of parasites usually exhibit complex life cycles, such as helminths and haemoparasites (Benesh *et al.* 2014; Atkinson and van Riper III, 1991). Helminth parasites (e.g. nematode, cestodes, acanthocephalan and trematodes) normally require the presence of several intermediate hosts and a definitive host to complete their life cycles (Duignan, 2001; Born-Torrijos *et al.* 2016; Benesh *et al.* 2014). In general the life cycle of helminth parasites comprise three stages: egg, larvae (which can undergo several moults depending on the species) and adult (Castro, 1996). Eggs and larvae are the free-living stages in the aquatic or terrestrial environment and, simultaneously, they are the infectious stages that infect intermediate hosts to pursue their development. The adult stage requires a definitive vertebrate host to reach maturity and for sexual reproduction (Chubb *et al.* 2010). Transmission may occur by ingestion, skin penetration or trophic transfer (Parker *et al.* 2003; Chubb *et al.* 2010). During the free-living stages, helminth parasites are susceptible to environmental conditions in the habitats they inhabit (aquatic or terrestrial) for which they had to develop protection measures (cuticles and capsules) to deal with the natural (e.g. temperature, salinity) and anthropogenic factors (e.g. pollutants) they are exposed to (Pietrock and Marcogliese, 2003; Thieltges *et al.* 2008).

Haemoparasites (such as haemosporidians, kinetoplastids, spirochaetales and filarial nematodes) use the blood cells of their bird hosts to feed and reproduce (Atkinson and van Riper III, 1991; Vanstreels *et al.* 2016). They depend on a blood-sucking arthropod vector (e.g. ticks and fleas) to facilitate transmission between hosts and therefore the distribution of haemoparasites often reflects that of their vectors (Quillfeldt *et al.* 2011). Within the vector, haemoparasites colonize the gut and salivary glands to undertake sexual and/or asexual reproduction (Atkinson and van Riper III, 1991; Dantas-Torres *et al.* 2017). Haemoparasites colonize the organs (e.g. liver, kidneys, lymphoid tissues, bone marrow, dermis) and blood tissues (erythrocytes and extracellular) of the vertebrate host after it was inoculated by the vector (Allison *et al.* 1978; Vanstreels *et al.* 2016).

2.2 Host factors

Several host-related factors can influence parasite diversity and abundance. Some of them include host body size and morphological dimensions of bill, claws and feathers (Clayton and Walther, 2001; Krasnov, 2008). Large bodies can sustain more parasites (Rózsa, 1997), while morphological structures can aid in the control of ectoparasites (e.g. preening with an overhanging upper beak is more effective as seen for Peruvian birds infested by chewing lice) (Clayton and Walther, 2001). Host sex and age are also important (Krasnov, 2008). Male hosts tend to harbour more parasites than females, because higher levels of androgens in males can lead to immunosuppression (Cohn, 1979; Folstad and Karter, 1992), while younger birds tend

to exhibit higher parasite loads compared to adults due to a more immature immune system to cope with infections (Buehler *et al.* 2009, Yabsley *et al.* 2012). Host behavioural traits, such as preening, sunning and dust bathing, can help to reduce parasite loads (Rothschild and Clay, 1952; Clayton *et al.* 2010). Studies on several seabird species (e.g. Guanay Cormorants (*Phalacrocorax bougainvillii*) recorded an increase in parasite-control behaviour in response to high infestations of Argasid ticks (*O. amblus*) (Duffy, 1983). Host behaviour in terms of habitat selection can also contribute to parasite infestation, as seen for the different parasite species infecting canopy birds (e.g. blood parasites), compared to those infesting ground birds (e.g. ticks) (Clayton and Walther, 2001). Host diet can also influence the occurrence of parasite species (Marcogliese and Cone, 1997). For example, the nematode *Contracaecum* spp. infect reed cormorant (*Phalacrocorax africanus*), great cormorant (*Phalacrocorax carbo*), Oriental darter (*Anhinga melanogaster*) and grey heron (*Ardea cinerea*) through the consumption of prey species (cichlid fishes and carp) (Barson and Marshall, 2004).

Host density is one of the most documented factors affecting parasite abundance (Duffy, 1983; Ramos and Drummond, 2017). High host population density provides resources (food and shelter) that facilitate parasite abundance and transmission within the host population (Brown and Brown, 1986; Duffy, 1988). Soft ticks (such as *Ornithodoros* spp.) may be a particular problem in high-density seabird colonies (Rothschild and Clay, 1952; Duffy, 1983; Duffy, 1988). For example, *O. amblus* infestations were particularly high in large and densely populated Guanay cormorant (*Phalacrocorax bougainvillii*), Peruvian booby (*Sula variegate*) and Peruvian brown pelican (*Pelecanus occidentalis thagus*) colonies (Duffy, 1983). In penguins, colony size was positively related to hard tick infestation (*I. uriae*) of king penguins (*Aptenodytes patagonicus*) (Mangin *et al.* 2003). This pattern is not only related to ticks as empirical studies have demonstrated a similar response for American swallow bugs (*Oeciacus vicarius*) in a cliff swallow colony (*Petrochelidon pyrrhonota*) (Brown and Brown, 2004). Heavier parasite burdens and a great variety of gastrointestinal helminth parasites has been found in wild bobwhites (*Colinus virginianus*) living in high density areas compared to those in lower density areas (Kellogg and Prestwood, 1968). Moreover, certain parasite species, such as the Argasid tick (*O. amblus*), have a broader host preference (i.e. more generalist) and benefit from colonies comprising of multiple bird species (Duffy, 1983; Duffy, 1988). Therefore, the presence of several seabird species in the same colony can also contribute to an increase in host density.

Host immune-competence is another important factor that can influence parasite infestations. Most of the parasites have evolved with their natural hosts (Poulin and Mouillot, 2004; Mans *et al.* 2008) and therefore the immune system is generally adapted to the normal

parasite burdens so the parasites do not adversely affect the hosts (Mitchell, 1991). However, stress conditions such as food shortage and pollution can affect the integrity of the host's immune system. A weaker immunity can facilitate infestations by common and novel parasite taxa (Borgsteede, 1996; Carrera-Játiva *et al.* 2014).

2.3 Micro- and macro-environmental factors

Depending on the life history characteristics of a parasite, the off-host environmental conditions may be more or less important for its survival, development and reproduction (Cantarero *et al.* 2013). Some parasites, such as permanent ecto- and endoparasites (e.g. lice, gastrointestinal helminth and haemosporidian parasites), depend on the microclimate provided by the host body to develop part or their entire life cycle (Marshall, 1981; Kuhn *et al.* 2016). In lice for example, the host body offers more constant temperature and moisture ranges compared to the external environment (Marshall, 1981). However, parasite survival can also depend on the variation of abiotic factors at different parts of the host body to which they are adapted. For example, the temperature at the base of the feathers located under folded wings is higher than at exposed perimeters. Thus, each body area offers the conditions for lice adapted to the specific microclimate to occur (Tompkins and Clayton, 1999).

Parasite taxa with free-living stages are also dependent on the microclimate offered by the off-host environment. In particular, the microclimate within the host nest can offer ideal microclimatic conditions that facilitate the development of parasites and promote parasite infestation (Vial, 2009). The microclimate within the bird nest is the result of a combination of factors such as the physical presence of the host (e.g. body heat and moisture from the respiration and excrement; Rothschild and Clay, 1952) and the characteristics inherent to the nest (e.g. location, shape, content and size) (Marshall, 1981; Cantarero *et al.* 2013). For example, the humidity within the hole-nests of passerines have been found to influence the survival and reproduction of fleas (Heeb *et al.* 2000) and a study on European starlings (*Sturnus vulgaris*) recorded that the presence of macro- and microparasites are related to the type of nest material in the nest (e.g. dry grass, leaves, barks, branches and herbs) (Gwinner and Berger, 2005). Furthermore, the opening of the nest (open cup, closed cup or cavities) was found to be a good indicator of haemoparasite (e.g. *Plasmodium* spp.) infection in Afrotropical birds (e.g. birds living in evergreen forests and riparian forest/woodland habitats) and differences in parasite infection rates were related to traits such as nest height, nest location, nest type and flocking behaviour (Lutz *et al.* 2015). Lastly, studies on the marsh tit (*Parus palustris*), great tit (*Parus major*) and blue tit (*Parus caeruleus*) suggested that the material used to build

artificial nest boxes can promote an environment that is conducive for ectoparasite infestations (Hebda and Wesołowski, 2012).

External environmental conditions such as vegetation structure, soil composition and local climatic conditions can also influence the development and survival of terrestrial parasites (Arriero *et al.* 2008; Macko *et al.* 2016). Some environmental factors that affect ectoparasites include temperature, solar radiation, rainfall and humidity (Sonenshine, 1993; Merino and Potti, 1996; Krasnov *et al.* 2002). The impact of these factors on parasite diversity and survival depend on the parasite species (Marshall, 1981; Merino and Potti, 1996). For example, the abundance of Ixodid ticks infesting passerines showed a seasonal variation, with high prevalence in winter months (lower ambient temperature and higher rainfall) compared to summer months (warmer and drier ambient) (Oorebeek and Kleindorfer, 2008). Conversely, Argasidae ticks seem to tolerate and can be abundant under higher temperatures compared to Ixodid ticks (critical temperature: Argasidae ticks 62-75 °C; Ixodidae ticks 32-45 °C) (Lees, 1947). Even ectoparasites with no free-living stages can be affected by the local climatic conditions (Johnson and Clayton, 2003). For example, a decrease in survival of chewing lice (genus *Dennyus*) was experimentally shown when reducing ambient temperature and increasing relative humidity (Tompkins and Clayton, 1999). The prevalence of haemoparasites can be indirectly affected by climatic conditions due to the effect of climate on the distribution and abundance of their arthropod vectors (Jones and Shellam, 1999; Furuno *et al.* 2017). For example, Zamora-Vilchis *et al.* (2012) recorded a positive relationship between the prevalence of haemoparasites (genera *Plasmodium*, *Haemoproteus*, *Leucocytozoon* and *Trypanosoma*) in birds and temperature in lowland areas of the Australian Wet Tropics bioregion. The authors ascribe this relationship to the abundance of vector species in the lowland areas. Finally, parasites in the marine environment face their own challenges. Free-living stages (e.g. eggs and larvae) must deal with abiotic factors such as water temperature, depth, salinity, oxygen and hydrogen ion concentration (pH) (Pietrock and Marcogliese, 2003; Kuhn *et al.* 2016). For example, an increase in water temperature can increase the proliferation and release of trematode cercariae from their intermediate host (snail) (Poulin, 2006), while the decrease of water salinity (dilution) can reduce the infectivity and survival of the larvae and eggs of marine parasites (Pietrock and Marcogliese, 2003).

3. The endangered African penguin, its conservation and parasites

Despite being important components of biodiversity, parasites and diseases can be serious threats to animal health (Daszak *et al.* 2000). This is especially relevant for small and endangered wildlife populations, where the negative impacts of parasites can increase the risk

of species extinctions (Hudson *et al.* 2006; Robinson *et al.* 2010). In South Africa, one of the most threatened seabird species is the African penguin (*Spheniscus demersus*). This species has experienced a severe population decline (>50%) over the last three generations. As a result, the conservation status of the species is currently listed on the IUCN Red list as Endangered (BirdLife International, 2016). It has declined by >90% of historically recorded levels and continues to decline at most of the colonies. The overall breeding population decreased from ca. 63,000 pairs in 2001 to ca. 26,000 pairs in 2009 (Kemper *et al.* 2007; Crawford *et al.* 2011), while it reached 25,000 pairs in 2015 (BirdLife International, 2016).

3.1 Generalities on the African penguin

The African penguin is the only penguin species that breeds along or on the African continent (Shelton *et al.* 1984), and is endemic to southern Africa (Crawford *et al.* 2011). The species currently breeds in 24 islands and 4 mainland colonies from the west coast of Namibia to the east coast of South Africa (Crawford *et al.* 2013). The establishment of penguin colonies has been associated with the distribution of their main prey: the Cape anchovy (*Engraulis encrasicolis*) and the South African sardine (*Sardinops sagax*) (Crawford *et al.* 2006). African penguins frequently hunt in synchronized flocks with a diurnal foraging rhythm (Siegfried *et al.* 1975). They are central place foragers with a feeding area within 20 km of a breeding colony (Waller, 2011). During the breeding season (the timing of breeding varies around the southern African coast but the breeding season is extended normally from February to September/October; Crawford *et al.* 1995; Crawford *et al.* 2006), adults stay at the nest after the day's foraging trip, i.e. at dawn, late afternoon or early evening (Cooper, 1980). Adults breed for the first time when they are approximately 4 years old and they usually lay two eggs (Williams and Cooper, 1984; Crawford *et al.* 1999). Both parents participate in the incubation process, which takes around 40 days (Williams and Cooper, 1984). Chicks remain in the nest and depend exclusively on their parents to be fed and kept warm until they are around 26-30 days old. After that, both parents leave the nest to feed and come back sporadically, leaving the chick mostly unattended (Seddon and van Heezik, 1991). Chicks remain at or near their nest until they are approximately 80 days old (this can range between 60-120 days) when they become independent (Williams and Cooper, 1984). African penguins make above ground open nests (superficial open nests) or dig burrows where they mate, incubate eggs and guard chicks. The nests are built in shaded areas under vegetation, in holes or burrows (Frost *et al.* 1976). In addition, artificial nests (e.g. cement, wood and fiberglass) have been deployed at multiple colonies in order to sustain a suitable nesting habitat (Kemper *et al.* 2007; Sherley *et al.* 2012; Pichegru, 2013). Adult penguins show high fidelity to their breeding colonies and nest sites

once they have bred (Whittington *et al.* 2005). As a seabird species, the African penguin has a relevant role in the marine and terrestrial ecosystems. African penguins are among the top predators in the ocean, and as such, they can indicate availability and productivity of marine resources (Cairns, 1988). Likewise, they can modify community composition in terrestrial vegetation by altering physical and chemical soil properties and seed dispersal (Ellis, 2005). African penguins are sensitive to habitat alterations (e.g. pollution and human disturbance) and therefore can indicate changes in their environment (Boersma, 2008; Parsons *et al.* 2008; Waller, 2011). They also have a social impact since they contribute to local economy (e.g. tourism or the use of guano as fertilizer) (Shelton *et al.* 1984; Lewis *et al.* 2012). This charismatic seabird is a threatened animal species in South Africa. Among the various legislations that aim at reducing the impact of threats this species faces, a national management plan for the African penguin was compiled and gazetted (Department of Environmental Affairs, 2013). This document coordinates the efforts of various organizations (governmental, non-governmental and international) and describes specific actions to recover the African penguin population. Among its actions, it encourages research on the development of appropriate artificial nests to improve breeding habitat.

3.2 Threats to the African penguin conservation

Several factors may have exerted stress on some or all of the penguin colonies, which may explain the widespread decline of penguin population. Potential stress factors include: over-exploitation of eggs for human consumption (this practice was common in the 1900s) (Rand, 1969), habitat degradation (e.g. removal of guano and thus less available breeding sites) (Frost *et al.* 1976), pollution (e.g. oil spills) (Wolfaardt *et al.* 2009), human disturbances (e.g. tourism) (Lewis *et al.* 2012), climatic conditions (e.g. heat stress) (Waller, 2011), natural predators (e.g. kelp gulls *Larus dominicanus*) (Frost *et al.* 1976;), introduced predators (e.g. rats, cats, dogs) (Department of Environmental Affairs, 2013), and decreases in fish stocks that form the basis of their nutrition along the South African coastline (Crawford *et al.* 2011).

Other contributing factors that could lead to the species' decline include high parasite infestations (Duffy and Daturi, 1987) and disease outbreaks (Yabsley *et al.* 2012). However, as yet, limited empirical data is available on the importance of parasites and pathogens in this species (Duffy and Daturi, 1987; Department of Environmental Affairs, 2013). A diverse set of parasite taxa are associated with African penguins and include trematodes (*Cardiocephaloides physalis*, *Renicola sloanei*, *Metorchis* spp. and *Gigantobilharzia* spp.) (Heinemann, 1936; Randall and Bray, 1983; Horne *et al.* 2011; Aldhoum and Horne, 2015), cestodes (*Tetrabothrius* spp.), nematodes (*Contracaecum* sp., *Contracaecum variegatum* and *Cyathostoma phenisci*)

(Kanarek *et al.* 2013; Viljoen, 2015; Parsons and Vanstreels, 2016), haemoparasites (*Plasmodium* sp. *Babesia piercei*, *Leucocytozoon tawaki*, *Borrelia* spp. and *Anaplasma* (*Candidatus* Anaplasma sphenisci)) (Brossy, 1992; Earlé *et al.* 1992; Graczyk *et al.* 1995; Brossy *et al.* 1999, Yabsley *et al.* 2012, Vanstreels *et al.* 2018), and ectoparasites such as chewing lice (*A. demersus*; Banks and Palma, 2003), fleas (*P. humboldti*; Parsons and Vanstreels, 2016) and soft ticks (*O. capensis*; Daturi, 1986; Duffy and Daturi, 1987).

The various stress factors are scale dependent with climate change affecting all or most colonies. It is anticipated though that given the high mobility of the species, penguins will migrate from high to low stressed colonies. If this is the case, then low stressed colonies may experience a gradual increase in resident penguin numbers, which may result in increased pressure on local resources such as nesting sites and food, as well as lead to higher bird and nest densities. The latter in particular can have detrimental effects on density-dependant parasite transmission (Tella, 2002; Ramos and Drummond, 2017)

4. Problem statement

In recent years, most African penguin colonies have experienced a decrease in population size with only a few colonies staying constant or slightly increasing. This pattern is specifically relevant to the colonies along the south-western coast of South Africa. For example, for the period 2010-2017 the number of breeding pairs at Dassen Island decreased substantially from 4929 to 1922, while a slight decrease was recorded for Simon`s Town (known previously as Boulders Beach) from 933 to 854 over the same period (CapeNature, DEA and SANParks unpublished data). In contrast, there was a large increase in the number of breeding pairs from 466 to 1774 at the Stony Point colony at Betty's Bay (CapeNature unpublished data). With regard to the latter, during the past few years conservationists recorded nest and chick abandonment in the Stony Point colony as well as ticks on penguin chicks and around nests. It is therefore surmised that the gradual increase in breeding pairs might facilitate an increase in ectoparasite infestations (particularly soft ticks) in and around penguin nests and that, the parasites are one of the driving factors behind nest abandonment in the colony. However, it is at present uncertain if Stony Point has higher parasite infestations compared to other colonies. In addition, if parasite infestations are in fact higher at Stony Point, it is unknown whether the pattern of infestation is random or related to specific factors such as nest type (natural vs. artificial) or spatial position of nests within the colony. To date, few studies have assessed the factors that drive parasite infestation in or on African penguins or in their nests. A study by Daturi (1986) investigated the microclimatic conditions that influence soft tick abundance in the nests at the Marcus Island colony on the west coast of South Africa. Although several of

the parameters used in the study were qualitative as opposed to quantitative it does suggest that level of nest opening (open vs. closed) and nest occupancy (deserted vs. occupied) affects tick abundances in nests. In addition, the few clinical studies that have been conducted on African penguins were based on data obtained from animals kept at rehabilitation centres (Southern African Foundation for the Conservation of Coastal Birds, SANCCOB). Consequently, little is known about the parasite diversity and level of infestation on penguins and in their nests in natural settings. As such, it is uncertain if there are between-colony differences in parasite infestations and if so what the potential driving factors are for these differences.

5. Aims and predictions

The main aim of this project is to record the parasite diversity and infestations on African penguins and in their nests at the Stony Point colony and to establish the potential effect of parasite infestations on penguin health. This project has direct implications for the management of African penguins at the Stony Point colony and the conservation of the endangered species in general.

The objectives of this study are:

1. **Identify the on-host and in-nest parasite infestations (richness, abundance and prevalence) across different colonies along the south-western coast of South Africa, and determine the role of host (host age and density) and environmental characteristics (colony location and climatic factors) on parasite loads.** It is predicted that parasite infestations will vary between colonies. Specifically, parasite infestations will be higher in colonies with high nest density compared to colonies with low nest density. It is also predicted that parasite infestations will vary with age and that penguin chicks will have higher infestation compared to adult penguins due to the fact that chicks have a weaker immune response compared to adults. This objective is addressed in chapter 2.

2. **Determine the potential effect of parasites on the health status and condition of African penguins.** It is predicted that parasites will exert a negative effect on penguin health and that the effect will be reflected in clinical parameters out of the normal range described for the species. This objective is addressed in chapter 3.

3. **Establish the potential effect of nest characteristics (nest type, occupancy, location and orientation) and microclimatic conditions (soil temperature, soil moisture and nest material moisture) on the abundance and prevalence of ectoparasites in the nest and**

on the health condition of penguins at the Stony Point penguin colony. It is predicted that the prevailing microclimatic conditions that is associated with the nest will influence parasite infestations in the nest. Specifically, it is predicted that parasite infestations will be higher in artificial compared to natural open nests. This may be due to the fact that artificial nests provide a more stable microclimate for parasite development compared to exposed natural nests. We expect that the condition of penguins in artificial nests will be negatively affected compared to penguins in natural nests. This is addressed in chapter 4.

4. **Assess the efficiency of a modified Berlese funnel system as a quantitative method to extract ectoparasites from African penguin nests**. It is predicted that the funnel method will not be as effective as total counts in recording the abundance and prevalence of ectoparasites from nest samples. This is addressed in chapter 5.

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Chapter 2

Parasite diversity associated with African penguins (*Spheniscus demersus*) and the effect of host and environmental factors

(Currently in review in *Parasitology*)

Abstract

The African penguin (*Spheniscus demersus*) is a critically endangered species endemic to southern Africa. Limited information is available on the parasite diversity associated with the species in natural settings. This study explored the diversity and incidence of parasites associated with African penguins and their nests, and recorded the effect of host and environmental factors on parasite infestation. Ecto-, haemo- and helminth parasites were recorded from 210 adult birds, 583 chicks and 628 nests across five colonies (two mainland and three islands) along the south-western coast of South Africa, in 2016 and 2017. Mean nest density (total and active nests) and climate variables (temperature and precipitation) were obtained for each colony. *Parapsyllus humboldti* was the most abundant and prevalent ectoparasite on penguins and in nests (69.10% and 57.80%, respectively), while Piroplasmorida/Haemosporida (33.51%) and *Cardiocephaloides* spp. (56.17%) were the most prevalent haemo- and helminth parasites of penguins, respectively. In general parasite abundance and prevalence was significantly affected by penguin age (chicks vs. adults), location (mainland vs. islands), nest density (total and active nests) and season (spring vs. autumn/winter). It is concluded that parasite infestations are structured and that penguin chicks at mainland colonies are more at risk of parasite infestations during spring.

Key words: Ectoparasites; haemoparasites; helminth parasites; *Spheniscus demersus*; environmental factors; penguin colonies.

Introduction

Seabirds occupy diverse habitats that include marine and terrestrial ecosystems where they find resources to feed, nest, breed, find shelter and moult (Schreiber and Burger, 2001). In this dual environment, seabirds are exposed to parasites through their diet and eating habits (e.g. helminths present in fish species) (Randall and Bray, 1983; Brandão *et al.* 2014), nesting sites (e.g. ectoparasites in the nest material and soil) (Daturi, 1986; Smith *et al.* 2008) and through their interaction with other co-occurring congeneric and confamilial birds (e.g. bird-specific ectoparasites and pathogens such as viruses and bacteria) (McCoy *et al.* 2002).

There are several factors that can cause among colony variation in parasite diversity and level of infestations in seabirds (Jones and Shellam, 1999). Most seabird species form large colonies comprising up to thousands of breeding pairs (Schreiber and Burger, 2001) and therefore colony size and especially the nest density can facilitate high infestations of both host (lice) and nest parasites (fleas and ticks) (Rivera-Parra *et al.* 2014; Ramos and Drummond, 2017). For example, tick infestation has been found to be higher in Peruvian boobies (*Sula variegata*), a seabird that nest in large groups than in blue-footed boobies (*Sula nebouxi*), which tend to cluster at relatively lower densities (Duffy and Campos de Duffy, 1986). The presence of co-occurring congeneric and confamilial bird can further increase host and nest density, which can result is higher abundances and transmission of bird-specific parasites (Duffy, 1988). The level of parasite infestation in prey species can also vary spatially and this may influence the risk of infection for seabirds that act as definitive hosts (Van der Lingen *et al.* 2015; Weston *et al.* 2015; Levsen *et al.* 2018). A large scale study by Levsen *et al.* (2018) recorded regional difference in parasite infestations of Anisakid nematodes in sardine (*Sardinus pilchardus*) and other commercial fish species within the European fishing grounds. Several of these nematodes require seabirds as definitive hosts (Anderson, 2000). Climatic conditions on land and in the water can also affect parasite distribution. Nidicolous ectoparasite species (spend time in nests and shelters) are susceptible to mean temperature and precipitation (Sonenshine, 1993; Marshall, 1981*a*) while water temperature and salinity can effect most marine endoparasites in fish (Möller, 1978). In addition, bird age and immune status are also important factors (De Lope *et al.* 1998; Van Rensburg, 2010). For example, a study on the seabird Kittiwake (*Rissa tridactyfa*) recorded higher tick infestations on intermediate age chicks compared to younger and older chicks. The authors surmised that this may be due to the fact that intermediate aged chicks spend more time in the nests and are therefore more exposed to ticks (Boulinier and Danchin, 1996). Knowledge of the factors that drive parasite infestation is important as parasites can directly (cause stress, anaemia and reduced fitness) (Johnson and Clayton, 2003; Bitam *et al.* 2010) and indirectly (transmit disease-causing microbes such as protozoa, bacteria

and viruses) affect the condition and survival of their host (Nuttall, 1984). Although several studies have been conducted on parasites of seabird at the terrestrial-marine interface (e.g. Gauthier-Clerc *et al.* 1999; Frenot *et al.* 2001; Carrera-Játiva *et al.* 2014; Rivera-Parra *et al.* 2014), little is known about the factors that influence parasite loads on seabirds that naturally occur along the southern African coastline (Daturi, 1986; Duffy and Daturi, 1987).

In South Africa, seabirds inhabit a large sea border extending 3,000 km along the coast of the Atlantic and Indian Oceans, from the Orange River on the Namibian border to Ponta do Ouro on the Mozambique border (Heydorn, 1989). Some of the parasites associated with seabirds in southern Africa are typically associated with avian species across the world (Boyd, 1951; Atkinson and van Riper III, 1991; Poulin, 1999) and include protozoa, viruses, gastrointestinal helminth parasites and ectoparasites (Parsons and Vanstreels, 2016).

The African penguin (*Spheniscus demersus*) is endemic to the Benguela Upwelling Ecosystem (Crawford *et al.* 2011) and the only penguin species that breeds in Africa (Shelton *et al.* 1984). The species breeds in 28 colonies (24 islands and four mainland) (Crawford *et al.* 2013) distributed from central Namibia to the east coast of South Africa (Crawford *et al.* 2011). The suitability for breeding sites has been linked to the distribution and abundance of their primary prey: the Cape anchovy (*Engraulis encrasicolis*) and the South African sardine (*Sardinops sagax*) (Crawford *et al.* 2006). During the breeding season (in South Africa normally extended from February to September/October; Crawford *et al.* 1995; Crawford *et al.* 2006), the adults spend most of the day catching fish at sea and return to the colonies in the evenings to feed the chicks and relieve their mate (Cooper, 1980). They lay two eggs that are incubated for about 40 days. After hatching, the chicks are under parental care and become independent after ca. 80 days (Williams and Cooper, 1984). Historically, African penguins were common on nearshore islands, but more recently the species also started to occupy mainland areas. It is surmised that this movement may have been due to excessive harvesting of eggs (for human consumption) and guano (for fertilizer) on islands (Rand, 1969; Whittington *et al.* 1996), although a reduction in food resources along certain islands may have also contributed (Shelton *et al.* 1984; Crawford *et al.* 2011). Consequently, the species has suffered severe (>50%) and rapid population decline over three generations, mainly on island colonies, and its conservation status is listed as Endangered (BirdLife International, 2016). Studies conducted on parasites associated with African penguins have reported ectoparasites such as soft ticks (*Ornithodoros capensis*) (Daturi, 1986; Duffy and Daturi, 1987), louse (*Austrogoniodes demersus*) (Banks and Palma, 2003) and fleas (*Parapsyllus humboldti*) (Parsons and Vanstreels, 2016); haemoparasites (e.g. *Plasmodium relictum, Babesia piercei, Leucocytozoon tawaki*, *Borrelia* sp. and *Anaplasma* (*Candidatus* Anaplasma sphenisci)) (Brossy, 1992; Earlé *et al.* 1993;

Graczyk *et al.* 1995; Brossy *et al.* 1999; Yabsley *et al.* 2012; Vanstreels *et al.* 2018) and helmiths such as trematodes (*Cardiocephaloides physalis* and *Renicola sloanei*) (Randall and Bray, 1983; Horne *et al.* 2011), cestodes (*Tetrabothrius lutzi*, *Tetrabothrius eudyptidis*) and nematodes (*Contracaecum variegatum* and *Cyathostoma phenisci*) (Parsons and Vanstreels, 2016). All of the above mentioned studies on African penguins have mainly been conducted on animals that were sporadically admitted to rehabilitation centres due to illness or poor condition. Animals in poor body condition normally have elevated parasite infestations (Obendorf and McColl, 1980; Krasnov *et al.* 2005) and thus may not be representative of the parasite diversity and abundance in natural healthy populations. Further, studies on parasites associated with African penguin nests have been restricted to a single island colony, where only Argasid ticks (*O. capensis*) were recorded (Daturi, 1986; Duffy and Daturi, 1987). As yet, there is no empirical data on the parasites associated with African penguins and their nests across multiple colonies in South Africa. In addition, little is known with regard to the factors that drive among and within colony parasite infestations on African penguins and in their nests.

The aims of the study were: 1) to record the diversity and prevalence of parasites associated with African penguins and their nests at multiple colonies along the south-western coast of South Africa, and 2) to establish the effect of various host and environmental factors on parasite infestation patterns. We predict that penguin chicks will have higher parasite infestations compared to adults. This may be due to a combination of factors such as lower immunity and closer association with nests (and nest parasites) in chicks. We further predict that parasite abundance and prevalence will be positively related to nest density. Colonies with higher nest densities provide more resources (food and shelter) that can facilitate higher parasite infestations.

Materials and methods

Study site and design

The study was conducted at five African penguin colonies. Three islands: Dassen-, Dyer-, and Robben Island, and two mainland: Stony Point and Simon's Town (previously known as Boulders Beach) along the south-western coast of South Africa during 2016 and 2017 (Figure 2.1; Table 2.1). Penguins (adults and chicks: 20 day-old and older) and their respective nests were randomly selected and sampled at the peak of the first breeding season between May-July (autumn/winter, i.e. cold and rainy season) each year. In addition, penguins and their nests were sampled at one colony (Stony Point) during a second breeding season in October-November (spring, i.e. warm and dry season) during 2016. Sixty penguins (20 adults and 40 chicks) and 40 nests were sampled at each colony in autumn/winter each year. At Stony Point, 105 penguins

(22 adults and 83 chicks) and 109 nests were sampled in autumn/winter 2016, 103 penguins (8 adults and 95 chicks) and 81 nests were sampled in spring 2016, and 105 penguins (20 adults and 85 chicks) and 118 nests were sampled in autumn/winter 2017 (Table 2.1). Sampling was conducted during the day starting at 9:00 and ending at 16:00.

Parasite collection from penguins and nests

Each penguin (adult and chick) was examined for 8 minutes. Ectoparasites (fleas, lice and ticks) were collected by systematically brushing the plumage for 1 minute around the pelvic area using a soft brush. Ectoparasites that occurred on the face of the animals were also removed using forceps. Parasites were stored in 70% ethanol. A new brush and clean tweezers were used for each animal. A blood smear was made from a drop of blood collected from the dorsal aspect of the foot using a mechanical pipette attached to a 23-gauge needle. The blood smear was air-dried and fixed with methanol. Penguin chicks naturally defecate when handed. This allowed the collection of fresh faecal material, which were fixed in 10% formalin and kept cool until examination in the laboratory. Body mass (kg) was recorded for each penguin with a handheld electronic scale (25kg/50lb Sensation). Penguin nests were sampled for parasites by collecting 200 ml nest material (including soil) from the centre of the nest. Nest material was stored in plastic jars sealed with a lid and kept cool until further processing.

Parasite recovery and identification

Ectoparasites were extracted from the nest material using a modified Berlese funnel method (Southwood, 1978). In a sealed unit naphthalene moth balls (100g) were used as a repellent and hung above the nest material for 24 hours (Daturi, 1986). Thereafter, each nest material sample was systematically examined using a dissecting microscope. The latter method was included due to the ineffectivity of the extraction method to remove all parasites. Parasites recorded by the two methods were combined. Ectoparasites were identified morphologically using taxonomic reference keys (Bedford, 1934; Jordan, 1942; Von Keler, 1952; Arthur, 1963; Kohls *et al.* 1965; Segerman, 1995; Banks and Palma, 2003) and counted. Ectoparasite species were identified to species level and the life stage and sex was recorded.

Thin blood smears were stained using an Eosin-Methylene Blue stain (RapiDiff kit) and examined by detecting presence of haemoparasites in 150 fields per slide under a light microscope (Leica Microsystems, Wetzlar, Germany) at 100x magnification (Palinauskas *et al.* 2008; Valkiūnas *et al.* 2008). Haemoparasites were identified to order level (Piroplasmorida/Haemospororida and Spirochaetales) based on morphological characters (Earlé *et al.* 1993; Campbell and Ellis, 2007; Peirce and Parsons, 2012; Vanstreels *et al.* 2016).

Faecal material (1 gm) was examined for helminth eggs using qualitative techniques. Nematode and cestodes eggs were detected with the modified Wisconsin sugar flotation method (Nolan, 2006) (specific gravity of sugar solution >1.14). The sedimentation technique described by Hansen and Perry (1994) was used to detect trematode, acanthocephalans and any egg that did not float with the flotation technique. The helminth parasites were identified to genus level (Horne *et al.* 2011; Carrera-Játiva *et al.* 2014; Viljoen, 2015).

Nest density

Nest density was recorded by counting the total number of nests (non-active and active nests) and active nests only (nests containing eggs, chicks and/or adults) in a 15x15m quadrant during the autumn/winter season each year. Five quadrants were randomly selected each year at all colonies apart for Stony Point. At the latter colony 12 quadrants were selected each year.

Climate data

Data on the annual mean temperature (°C) and annual precipitation (mm) was obtained for each colony from WorldClim (Global Climate Data) using the function getData in the ´raster` package in R (Hijmans and van Etten, 2012). Remote censed data was selected due to the lack of local weather data at all the colonies.

Data analysis

To assess the effect of different parameters on parasite infestations, we considered the total number of parasites (i.e. parasites at all life stages) and we combined the two flea species found in this study in one group. Since flea larvae only occur in the nest and adult fleas are found in the nest and on the host, we considered analysis at each life stage only for this ectoparasite in order to record differences. Morphological differentiation between flea species at the larval stage is notoriously difficult (Krasnov, 2008) and as such the larvae of *P. humboldti* and *E. gallinacea* (recorded only at Dassen Island) could not be distinguished. Consequently, data on flea larvae from Dassen Island was not considered in the calculation of abundance and prevalence of total fleas (i.e. no available data).

The effect of penguin age (adult and chick), colony location (mainland and island) and colony (Dassen-, Dyer-, and Robben Island, Stony Point and Simon's Town) on parasite loads during the autumn/winter season were assessed using generalized linear models (GLMs). Where needed, the effect of penguin body mass (kg) and year (2016 and 2017) were corrected for in the models. Since parasite data was highly skewed due to an excess of zeros, parasite data was first modified by adding the value of 1, log-transformed and rounded (Changyong *et al.*

2014), followed by testing for overdispersion (GLM ´quasipoisson`). We then used zero-inflated negative binomial (to correct for data overdispersion) models using the zeroinfl function from the 'pscl' R package (Jackman, 2017) to model data on parasite abundance with excess of zeros. Whenever the model did not fit the data (e.g. residual of the model did not follow a normal distribution) abundance data was transformed into presence/absence and we used GLM with a binomial distribution (function glm()). Parasite prevalence was assessed with GLM with a binomial distribution. Since we aimed at assessing the effect of different factors on parasite infections, we presented the full models with all independent variables in the main text. However, we also performed backward model selection based on Akaike information criterion (AIC), using the function step() in R and compared the selected models with the corresponding full models using a Chi-square test. To compare cross-colony parasite mean abundance/prevalence in relation to nest density (total nest density and active nest density) and climatic factors (air temperature and precipitation) we used ANOVA and TUKEY HSD tests. The effect of nest density and climatic factors on parasite mean abundance (i.e. number of parasites of a particular species divided by the total number of hosts examined; Bush *et al.* 1997) and prevalence (i.e. number of infected hosts by a particular parasite species divided by the total number of hosts examined; Bush *et al.* 1997) was assessed using Pearson and Spearman correlation tests. Statistical analysis included Wilcoxon rank sum test and proportion test to compare parasite abundance and prevalence, respectively, between sampling seasons (autumn/winter and spring) at Stony Point. Seasonal differences in parasite prevalence were assessed using parasites from chicks because chicks were mainly sampled in the spring season at Stony Point and helminth parasites were only recorded for chicks. All statistical tests and plot design were conducted in R 3·4·3 (R Core Team, 2017).

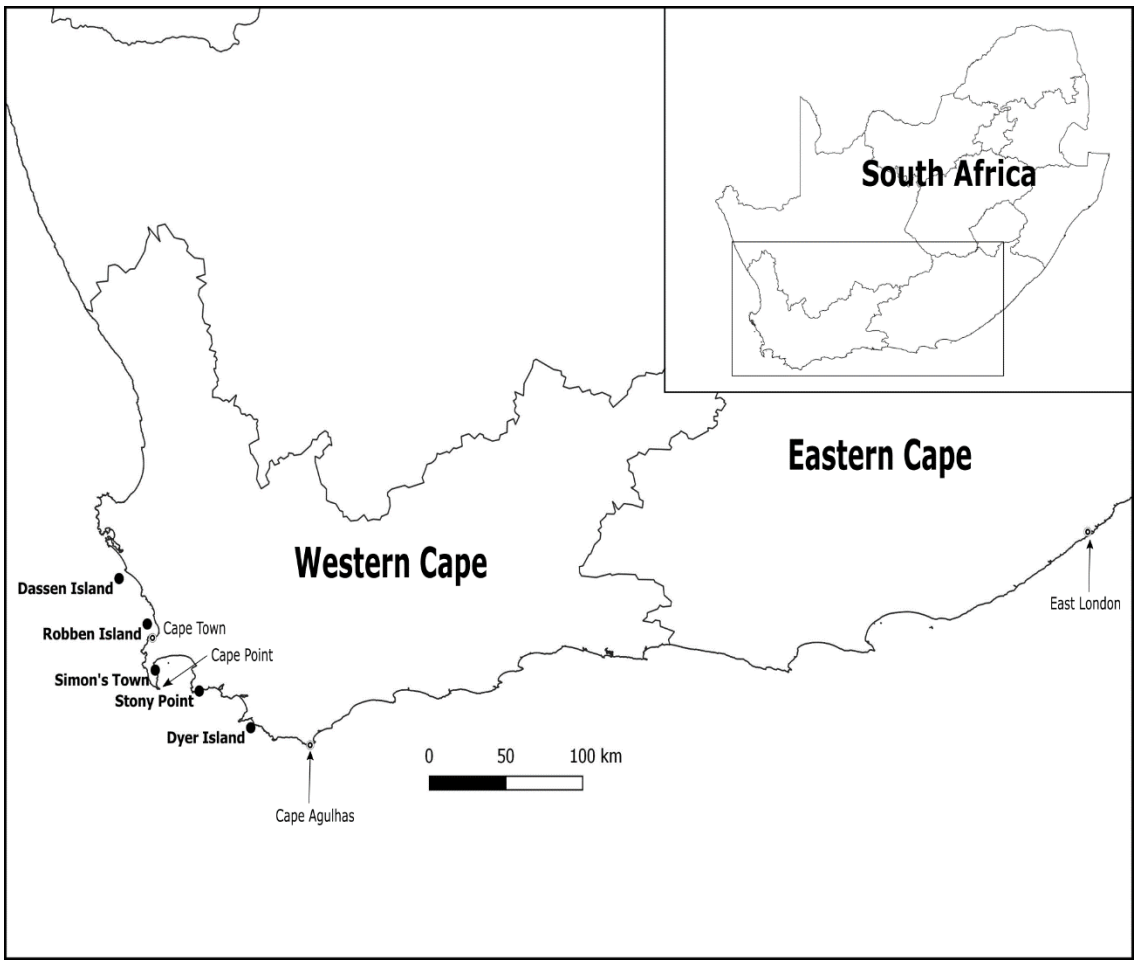

Figure 2.1. Map of the selected African penguin colonies along the south-western coast of South Africa. Two mainland (Simon's Town and Stony Point) and three island colonies (Dassen-, Dyer-, and Robben Island). Areas were plotted using GPS coordinates and QGIS open source Geographic Information System (http://qgis.osgeo.org).

Table 2.1. Locality, date of sampling, sample size, season and nest density at five African penguin colonies along the south-western coast of South Africa during 2016 and 2017.

Locality	Coordinates	Sampling date		Sample size (adult:chicks)	Season	Mean nest density (average/m^2) (total:active)
		2016	2017			
Island-based colonies						
Dassen Island	33.423647S, 18.086542E	12 May - 14 May	08 May - 12 May	120 birds (40:80) and 80 nests	autumn/winter	0.02:0.012
Dyer Island	34.684075S, 19.414769E	30 May - 01 June	29 July - 31 July	120 birds (40:80) and 80 nests	autumn/winter	0.08:0.06
Robben Island	33.807607S, 18.371231E	07 June - 26 June	29 May - 02 June	120 birds (40:80) and 80 nests	autumn/winter	0.05:0.007
Land-based colonies						
Stony Point	34.374151S, 18.895248E	29 June - 13 July	19 June - 12 July	210 birds (42:168) and 227 nests	autumn/winter	0.28:0.13
		24 October – 07 November		103 birds (8:95) and 81 nests	spring	
Simon's Town	34.197220S, 18.451285E	13 June - 20 June	26 June - 03 July	120 birds (40:80) and 80 nests	autumn/winter	0.22:0.14

Results

Three parasitic groups (ectoparasites, haemoparasites and helminth parasites) were recorded from 793 African penguins and 628 penguin nests at five colonies along the south-western coast of South Africa (Fig. 2.1, Table 2.2). Ectoparasites comprised of two fleas (*Parapsyllus humboldti* and *Echidnophaga gallinacea*), a louse (*Austrogoniodes demersus*) and a soft tick (*Ornithodoros capensis* s. s.). Haemoparasites were morphologically consistent with the orders Piroplasmorida/Haemospororida and Spirochaetales. Four helminth genera were detected in chicks (*Cardiocephaloides* spp., *Renicola* spp., *Contracaecum* spp. and *Cyathostoma* spp.). Prevalence, mean abundance, mean intensity (i.e. average number of parasites of a particular species divided by the number of infected hosts; Bush *et al.* 1997) and sex ratios of parasites associated with penguins, across the selected colonies, are provided in Table 2.3. Although mites (Acari) were recorded, they were mainly found in penguin nests and in high abundance. Mites are very specious and were not included in this study due to the taxonomic difficulty in distinguising between parasitic and non-parasitic soil mites (Proctor and Owens, 2000). However, the importance of mite data is recognised and will be included in follow-up studies.

Ectoparasites on penguins

The most abundant and prevalent ectoparasite on penguins was *P. humboldti* (4.57±0.2; 69.10%), while the second most abundant ectoparasite was *O. capensis* (s. s.) (0.51±0.07; 16.65%) of which larvae were mainly collected (Table 2.3). This life stage was the most abundant on penguins at three of the five colonies (Supplementary Table S2.1). *Parapsyllus humboldti* also exhibited the highest mean intensity on penguins (6.61±0.25), followed by *E. gallinacea* (4.95±0.60). The infestation level of *A. demersus* was in general very low, and apart from its absence at Simon's Town, no pattern was evident. However, the low abundance and prevalence of lice in the study could be due to a bias in the sampling design given that lice are small and difficult to detect. The sex ratios of the individual parasite taxa varied: *P. humboldti* recorded an equal sex ratio, *E. gallinacea* and *A. demersus* recorded a female-biased ratio, while only female *O. capensis* (s. s.) (three females infested three penguins) were recorded from penguins (Table 2.3).

Haemoparasites and helminth parasites of penguins

Piroplasmorida/Haemospororida was the most prevalent haemoparasite group (33.51%) compared to Spirochaetales (2.59%) in penguins. The helminth *Cardiocephaloides* spp. was the most prevalent genus (56.17%) in penguin chicks followed by *Contracaecum* spp. (12.83%) (Table 2.3).

Ectoparasites in nests

The prevalence, mean abundance, mean intensity and sex ratios of parasites recorded from penguin nests across the selected colonies are provided in Table 2.4. Only ectoparasites were recorded in nests, therefore the results are presented only for fleas and ticks. More than half of the nests were infested with *P. humboldti* (57.80%), of which the larval stage was the most abundant and prevalent (13.60±1.49; 47.29%). The second most abundant and prevalent parasite was *O. capensis* (s. s.) (6.37±1.90; 54.30%), of which nymphs were the most abundant and prevalent (2.5±0.94; 39.81%) (Supplementary Table S2.2). *Parapsyllus humboldti* also recorded the highest mean intensity, in infected nests, (25.30±2.52) of which larvae recorded the highest mean intensity (28.55±2.90), followed by *O. capensis* (s. s.) (11.74±3.48) of which tick larvae recorded the highest mean intensity (9.08±3.02) (Table 2.4). Ectoparasite taxa in nests exhibited different sex ratios: *P. humboldti* recorded a female-biased ratio, *E. gallinacea* recorded an equal number of males and females, and *O. capensis* (s. s.) recorded a male-biased ratio in nests (Table 2.4).

Factors that influence parasite infestations

The outcome of regression models showed a strong effect of penguin age, colony location and colony (Table 2.5). The majority of the full models used in the analysis did not show significant differences from the best models estimated with the AIC (Supplementary Table S2.3). In particular, abundance of total ectoparasites (*z*-statistic $p<0.001$), fleas (*P. humboldti* and *E. gallinacean* combined) (*z*-statistic $p<0.001$), and *O. capensis* (s. s.) (*z*-statistic $p<0.01$), and prevalence of Piroplasmorida/Haemospororida (*z*-statistic $p<0.001$) were significantly higher in chicks compared to adult penguins (Table 2.5). Interspecific variation in parasite infestations, on penguins, was recorded in mainland compared to island colonies. Penguins at mainland colonies recorded significantly higher abundances for total ectoparasites (*z*-statistic $p<0.001$) and fleas (*z*-statistic $p<0.001$) on penguins than island colonies. In addition, Piroplasmids/Haemospororida and *Cardiocephaloides* spp. were significantly more prevalent in penguins at mainland colonies compared to islands (*z*-statistic $p<0.001$ and *z*-statistic $p<0.01$, respectively). A similar pattern was recorded in penguin nests with significantly higher abundance recorded for total ectoparasites (*z*-statistic $p<0.01$), fleas (both life stages combined, *z*-statistic $p<0.01$), adult fleas (*z*-statistic $p<0.05$) and flea larvae (*z*-statistic $p<0.01$) at mainland colonies compared to islands.

Parasite infestations also varied between colonies with Stony Point and Simon's Town generally harbouring significantly more parasites on penguins and in nests. In particular, total ectoparasite abundance was significantly higher on penguins at Stony Point (*z*-statistic $p<0.05$)

and Simon`s Town (*z*-statistic p<0.01) than most of the other colonies (Table 2.5). In addition, at Stony Point a significantly higher *O. capensis* (s. s.) abundance (*z*-statistic p<0.05) and higher Piroplasmorida/Haemospororida prevalence (*z*-statistic p<0.05) were recorded for penguins compared to most or all other colonies. Penguin chicks at Stony Point also recorded a significantly higher prelavence of *Cardiocephaloides* spp. (*z*-statistic p<0.05) compared to other colonies, though when compared to Simon's Town the difference in prevalence was not significant. Abundance of *O. capensis* (s. s.) in nests were also generally higher at Stony Point compared to the other colonies and significantly so for Simon's Town (*z*-statistic p<0.05) and Dassen Island (*z*-statistic p<0.001). In contrast, penguins at Simon`s Town recorded a significantly higher abundance of fleas on penguins (*z*-statistic p<0.05) compared to other colonies. In addition, this colony recorded significantly higher infestations of total ectoparasites (*z*-statistic p<0.01), total fleas (both life stages combined) (*z*-statistic p<0.05), adult fleas *z*-statistic (p<0.05) and flea larvae in nests (*z*-statistic p<0.05) compared to the other colonies.

Cross-colony comparison of mean abundance and prevalence of parasites in autumn/winter in relation to nest density (total and active nest density) and climatic factors (temperature and precipitation) revealed siginificant differences across colonies for some parasitic groups (Piroplasmids/Haemospororida F=39.73, p<0.001; Cardiocephaloides spp. F=2.69, p<0.05; total parasites in nest F=4.12, p<0.01; fleas in nest F=9.605, p<0.001; total parasites on penguins F=14.27, p<0.001; fleas on penguins F=15.15, p<0.001) (Supplementary Figs. S2.1-S2.6). The infestation levels of several parasite taxa correlated with nest density (total and active). In particular, the prevalence of Piroplasmids/Haemospororida in penguins was significantly positively correlated with total and active nest density (r=0.97, p<0.01 and r=0.92, p<0.05, respectively) (total nest density as example Fig. 2.2A). The prevalence of *Cardiocephaloides* spp. in penguin chicks was significantly positively correlated with total nest density (Fig. 2.2B) (r=0.98, p<0.01). Likewise, mean abundance of total ectoparasite and *O. capensis* (s. s.) in nests was significantly positively correlated with the density of active nests (Fig. 2.2C) ($r_{Spearman}$=0.9, p<0.05) and total nests (Fig. 2.2D) ($r_{Spearman}$=0.9, p<0.05), respectively. Flea abundance in nests follow a similar pattern as the total ectoparasites in nests, however the response was not significant. The infestation levels of two parasite taxa correlate with climate. In particular, mean abundance of *A. demersus* on penguins was significantly negatively correlated with annual mean ambient temperature (Fig. 2.3A) and annual precipitation (Fig. 2.3B) (r=-0.95, p<0.05 and r=-0.92, p<0.05, respectively), while prevalence of *Contracaecum* spp. in chicks was significantly positively correlated with annual mean ambient temperature (Fig. 2.3C) and annual precipitation (Fig. 2.3D) (r=0.97, p<0.01 and r=0.94, p<0.05, respectively).

Several parasite taxa exhibited seasonal variation in infestations on penguin chicks at Stony Point during 2016. In particular, *P. humboldti* was significantly more prevalent (91.6% and 77.1% respectively, $p<0.05$) and abundant (8.1±0.7 and 4.6±0.5 respectively, $W=2619.5$, $p<0.001$) on chicks in spring compared to autumn/winter. A similar, but stronger pattern was recorded for *O. capensis* (s. s.) prevalence (75.8% and 9.6% respectively, $p<0.001$)) and abundance (2.8±0.4 and 0.1±0.05 respectively, $W=1181.5$, $p<0.001$) on chicks (data for *P. humboldti* and *O. capensis* (s. s.) abundance not shown) (Fig. 2.4A). Spirochaetales were also more prevalent in penguins in spring compared to autumn/winter, but only marginally significant (11.6% spring and 2.5% autumn/winter, $p=0.05$) (Fig. 2.4B). Helminth parasite infestations in chicks were not affected by season (Fig. 2.4C). Prevalence of ectoparasites in penguin nests also varied significantly across seasons. *Parapsyllus humboldti* was significantly more prevalent (68.0% and 42.2% respectively, $p<0.001$) and abundant (8.14±0.7 and 4.6±0.5 respectively, $W=0.37273$, p-value<0.001) in nests during spring compared to autumn/winter. Likewise, *O. capensis* (s. s.) was significantly more prevalent (95.1% and 56.0% respectively, $p<0.001$) and abundant (13.9±5 and 1.7±0.3, respectively, $p<0.05$) in nests in during spring compared to autumn/winter (Fig. 2.5).

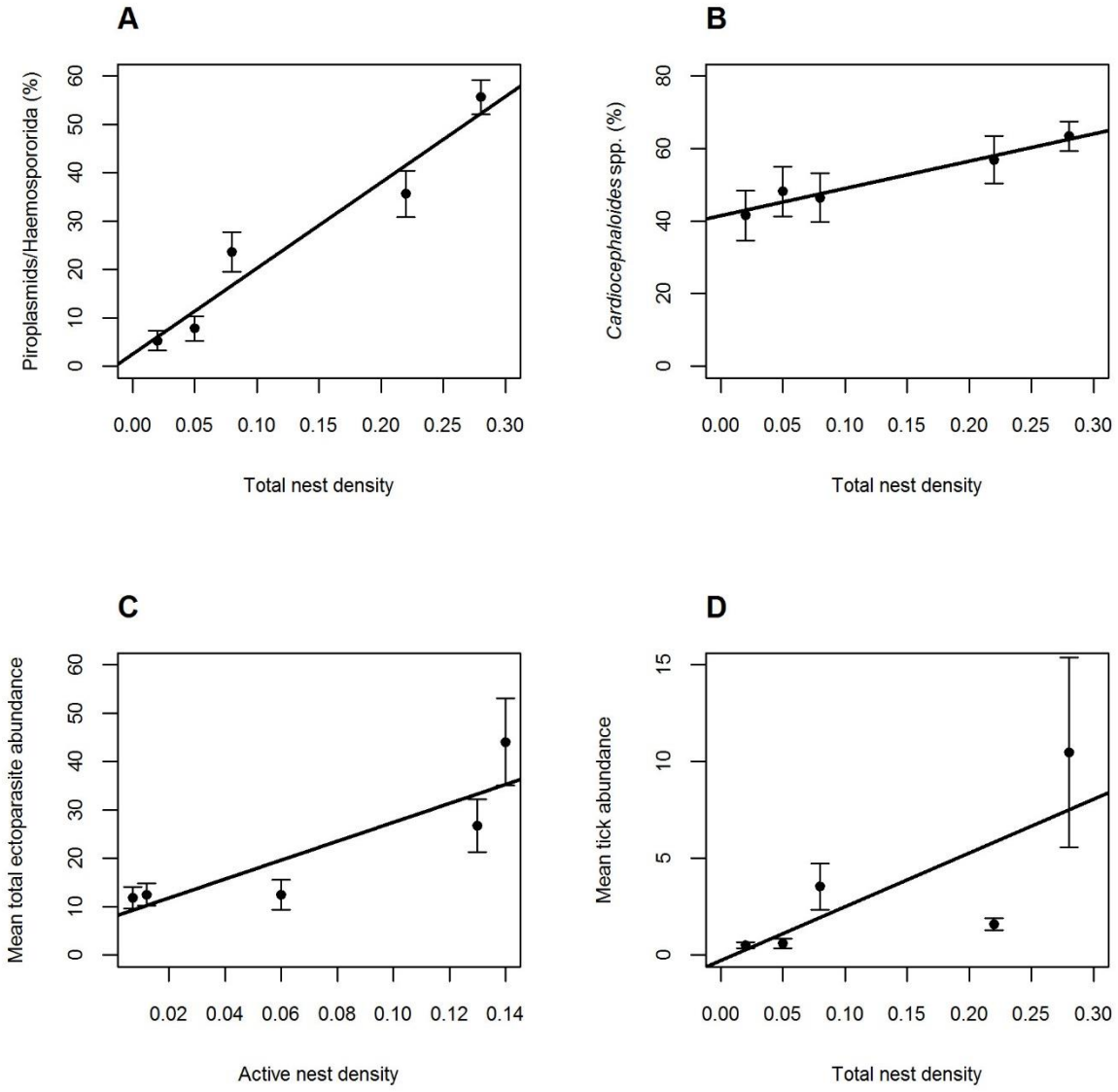

Figure 2.2. Pearson correlation between (A) Piroplasmids/Haemosporida prevalence and total nest density, and (B) *Cardiocephaloides* spp. prevalence and total nest density. Spearman correlation between (C) mean total nest ectoparasites and active nest density, and (D) mean nest ticks (*O. capensis* s. s.) and total nest density of African penguins.

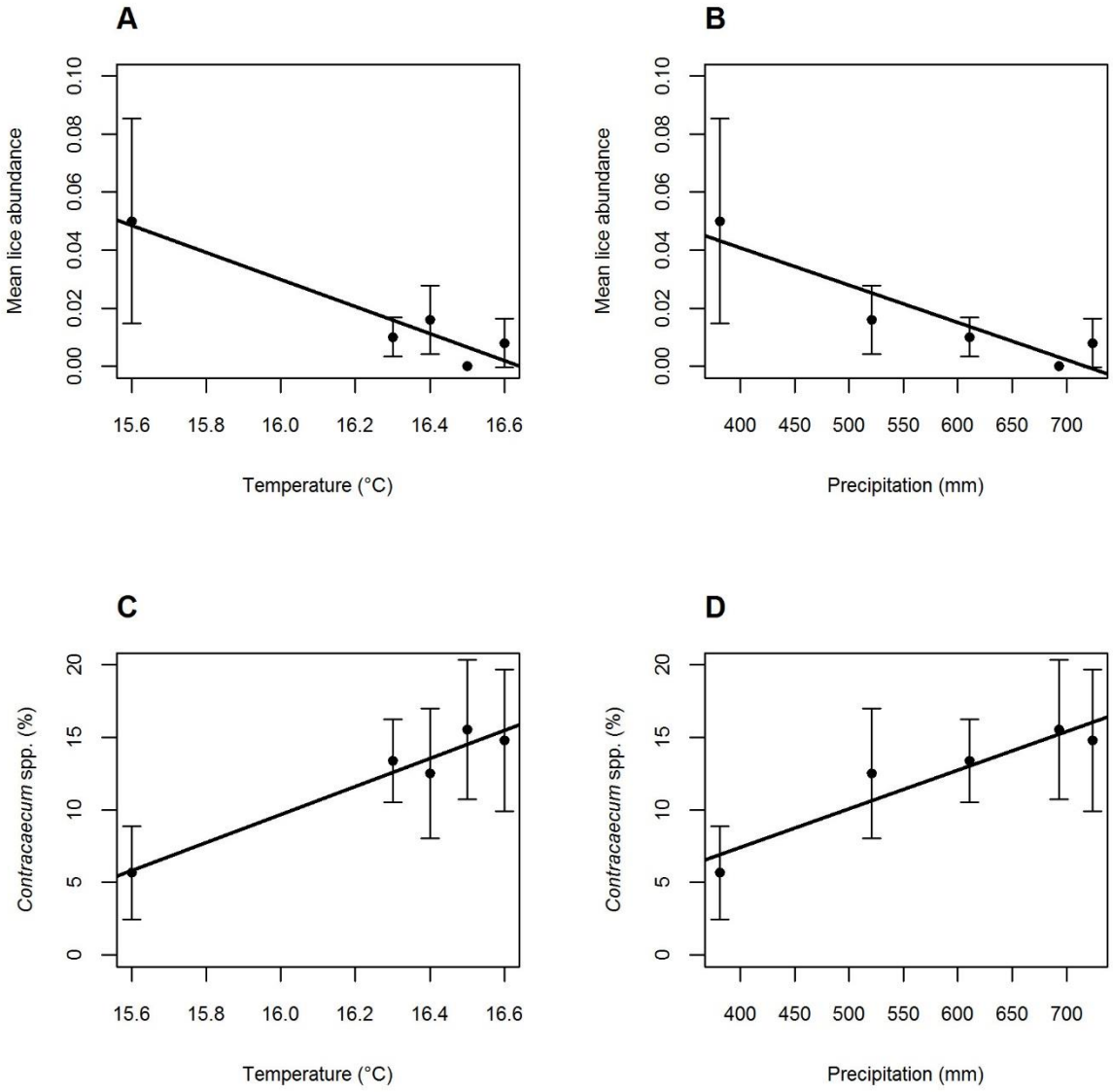

Figure 2.3. Pearson correlation between (A) mean abundance of lice (*A. demersus*) and annual mean temperature (B) mean abundance of lice (*A. demersus*) and annual precipitation, (C) *Contracaecum* spp. prevalence and annual mean temperature, and (D) *Contracaecum* spp. prevalence and annual precipitation at five African penguin colonies along the south-western coast of South Africa.

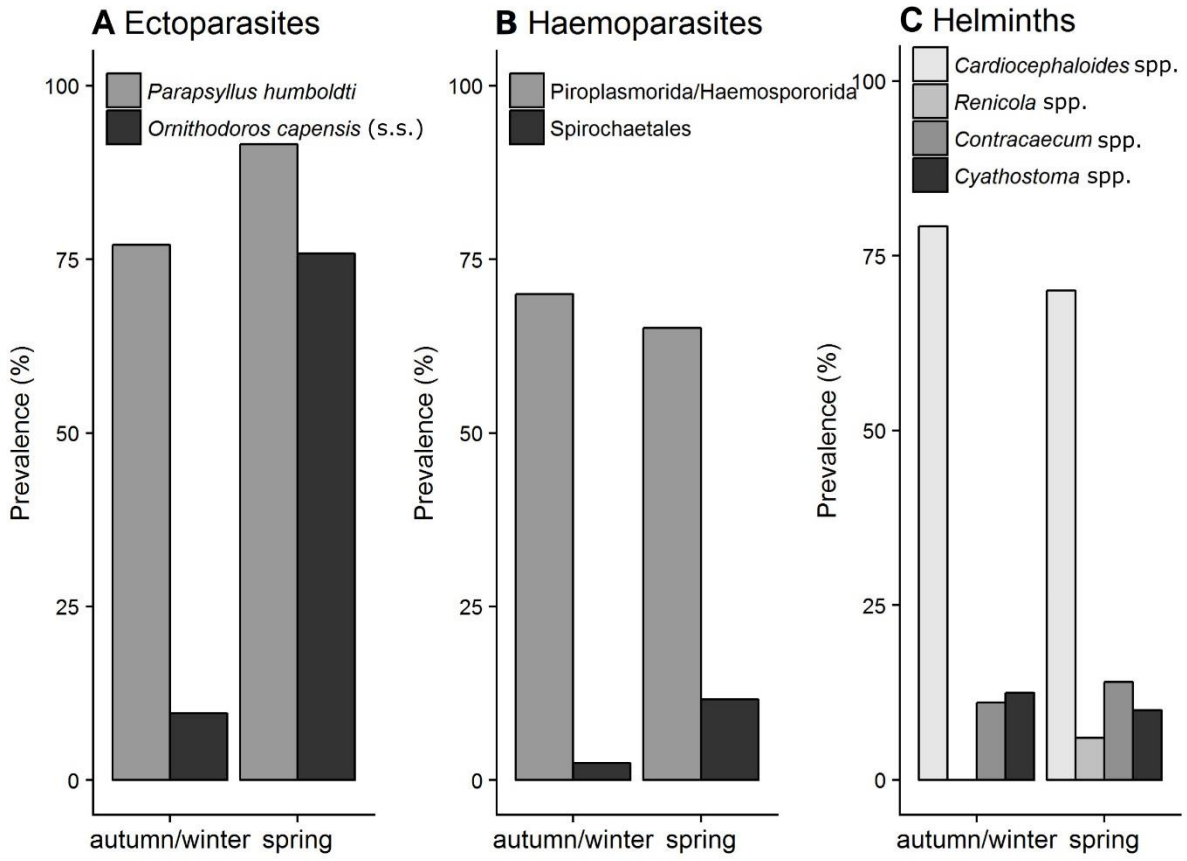

Figure 2.4. Prevalence of ectoparasites, haemoparasites and helminth parasites associated with African penguin chicks at Stony Point during two seasons (autumn/winter and spring) in 2016. Sample sizes N=178 (ectoparasites), 166 (haemoparasites) and 122 (helminths).

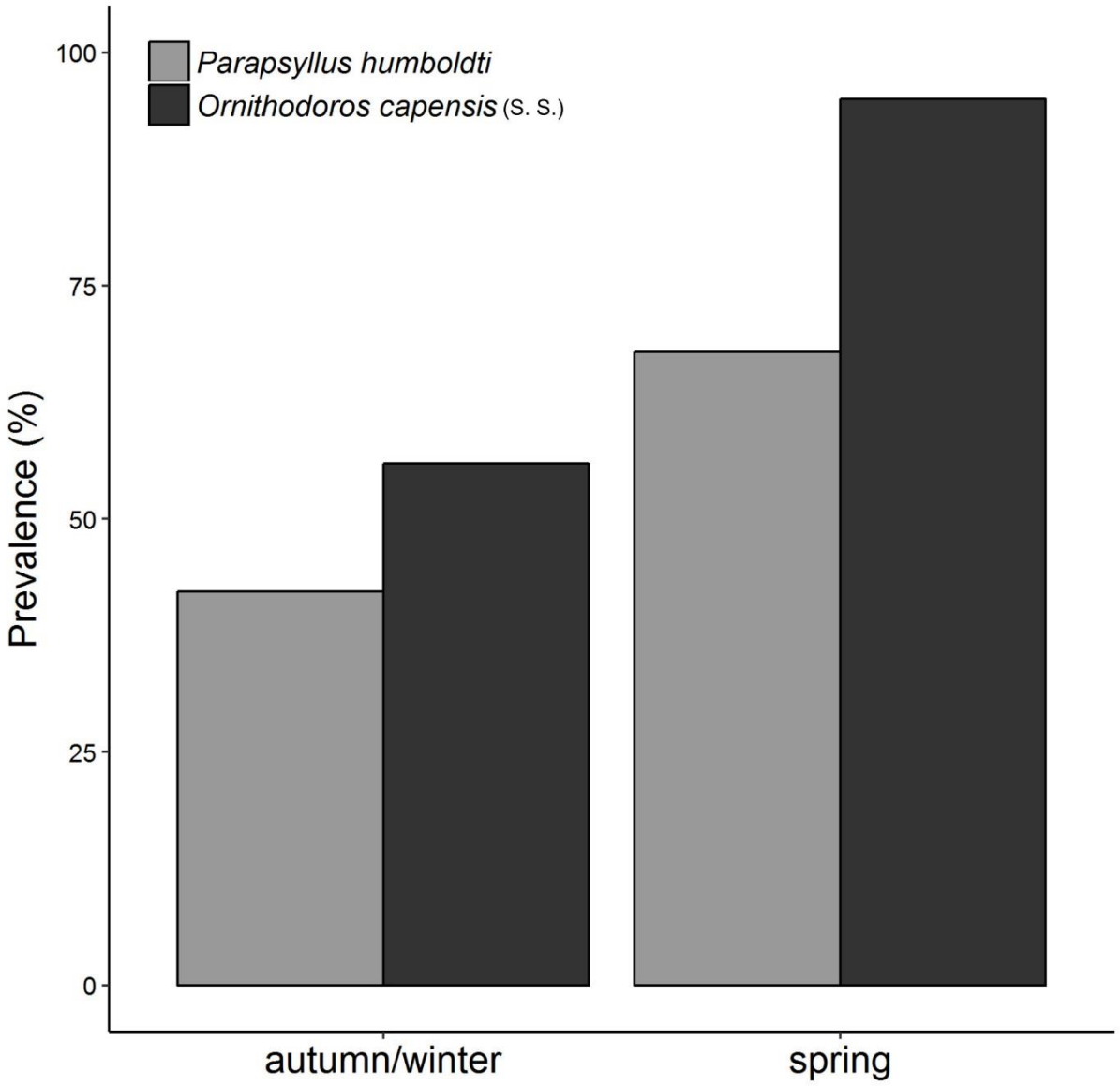

Figure 2.5. Prevalence of fleas and soft ticks in the nests of African penguins (N=190) at the Stony Point colony during two seasons (autumn/winter and spring) in 2016.

Table 2.2. Parasite taxa associated with African penguins and their nests at five African penguin colonies along the south-western coast of South Africa during 2016 and 2017.

	Order	Suborder	Family	Genus/species
Ectoparasites				
Fleas	Siphonaptera	Pulicomorpha	Rhopalopsyllidae	*Parapsyllus humboldti* (Jordan, 1942)
			Pulicidae	*Echidnophaga gallinacea* (Westwood, 1875)
Lice	Phthiraptera	Ischnocera	Philopteridae	*Austrogoniodes demersus* (Kéler, 1952)
Ticks	Parasitiformes	Ixodida	Argasidae	*Ornithodoros capensis* (s. s.) (Neumann, 1901)
Haemoparasites	Piroplasmorida (Wenyon, 1926)/ Haemosporida (Danilewsky, 1885) Spirochaetales (Buchanan, 1917)			
Helminths	Strigeidida		Strigeidae	*Cardiocephaloides* spp.
	Plagiorchiida	Xiphidiata	Renicolidae	*Renicola* spp.
	Ascaridida		Anisakidae	*Contracaecum* spp.
	Rhabditida	Strongylida	Syngamidae	*Cyathostoma* spp.

Table 2.3. Ectoparasites, haemoparasites and helminths recorded from African penguins at five colonies along the south-western coast of South Africa during 2016 and 2017. Sample sizes N=793 (ectoparasites), 734 (haemoparasites) and 413 (helminths).

Parasite species	Total prevalence (%)	Mean abundance (±SE)				Mean intensity (±SE)				Sex ratio (♂:♀)
		Larvae	Nymphs	Adults	Total	Larvae	Nymphs	Adults	Total	
Fleas										
P. humboldti	69.10[1]	0.001[2] (±0.001)	-	4.57 (±0.20)	4.57[1] (±0.20)	1[2]	-	6.61 (±0.25)	6.61[1] (±0.25)	1.08:1
E. gallinacea	5.30	-	-	0.26 (±0.05)	0.26 (±0.05)	-	-	4.95 (±0.60)	4.95 (±0.60)	0.16:1
Lice										
A. demersus	1.01	-	-	0.01 (±0.006)	0.01 (±0.006)	-	-	1.38 (±0.38)	1.38 (±0.38)	0.38:1
Ticks										
O. capensis	16.65	0.43 (±0.06)	0.08 (±0.03)	0.004 (±0.002)	0.51 (±0.07)	2.97 (±0.30)	2.25 (±0.75)	1	3.09 (±0.31)	only female
Haemoparasites										
Piroplasmorida/ Haemosporida	33.51	-	-	-	-	-	-	-	-	-
Spirochaetales	2.59	-	-	-	-	-	-	-	-	-
Helminths										
Cardiocephaloides spp.	56.17	-	-	-	-	-	-	-	-	-
Renicola spp.	0.73	-	-	-	-	-	-	-	-	-
Contracaecum spp.	12.83	-	-	-	-	-	-	-	-	-
Cyathostoma spp.	3.87	-	-	-	-	-	-	-	-	-

[1] Total prevalence, mean abundance and mean intensity of fleas excluded flea larvae from Dassen Island.

[2] Mean abundance and mean intensity of flea larvae excluded flea larvae from Dassen Island.

Table 2.4. Ectoparasites recorded from nests of African penguins (N=628) along the south-western coast of South Africa during 2016 and 2017.

Parasite species	Prevalence (%)				Mean abundance (±SE)				Mean intensity (±SE)				Sex ratio (♂:♀)
	Larvae	Nymphs	Adults	Total	Larvae	Nymphs	Adults	Total	Larvae	Nymphs	Adults	Total	
Fleas													
P. humboldti	47.29[1]	-	33.44	57.80[2]	13.60[1] (±1.49)	-	1.03 (±0.10)	14.62[2] (±1.54)	28.55[1] (±2.90)	-	3.08 (±0.26)	25.30[2] (±2.52)	0.7:1
E. gallinacea	-	-	0.64	0.64	-	-	0.01 (±0.005)	0.01 (±0.005)	-	-	1.5 (±0.29)	1.5 (±0.29)	1:1
Ticks													
O. capensis	21.34	39.81	30.57	54.30	1.94 (±0.66)	2.5 (±0.94)	1.94 (±0.67)	6.37 (±1.90)	9.08 (±3.02)	6.27 (±2.34)	6.35 (±2.16)	11.74 (±3.48)	1.63:1

[1] Prevalence, mean abundance and mean intensity of flea larvae excluded flea larvae from Dassen Island.

[2] Total prevalence, mean abundance and mean intensity of fleas excluded flea larvae from Dassen Island.

Table 2.5. Effect of colony location (mainland and island), colony (Stony Point, Simon's Town, Dassen-, Dyer- and Robben Island) and penguin age (adult and chick) on parasite infestation of African penguins and their nests during in the autumn/winter season (2016 and 2017). Type of analysis: Regression model ZINB (Zero-inflated Negative Binomial) and glm 'binomial' and proportion test. Significant values: ***=< 0.001, **=0.001- 0.01, *=0.01-0.05, ·=non-significant.

Type of Analysis	Dependent variable	Predictor	Intercept	Independent variables	Estimate	Standard error	z value	p-value
ECTOPARASITES ON PENGUINS								
ZINB	Total Ectoparasites	Location	Islands	Mainland	0.12738	0.032324	3.941	***
		Year	Year 2016	Year 2017	0.087586	0.032304	2.711	**
		Age	Adult	Chick	0.436554	0.05141	8.492	***
		Body mass			-0.003976	0.025458	-0.156	.
ZINB	Total Ectoparasites	Colony	Stony Point	Simon's Town	0.061695	0.04317	1.429	.
			Stony Point	Dassen Island	-0.104378	0.047471	-2.199	*
			Stony Point	Dyer Island	-0.327557	0.058797	-5.571	***
			Stony Point	Robben Island	0.006368	0.04372	0.146	.
		Year	Year 2016	Year 2017	0.099295	0.031331	3.169	**
		Age	Adult	Chick	0.444792	0.050382	8.828	***
		Body mass			-0.015403	0.024987	-0.616	.
ZINB	Fleas (*P. humboldti* and *E. gallinacea*)	Location	Islands	Mainland	0.117148	0.033258	3.522	***
		Year	Year 2016	Year 2017	0.068947	0.033196	2.077	*
		Age	Adult	Chick	0.42249	0.052495	8.048	***
		Body mass			0.007177	0.026195	0.274	.

ZINB	Fleas (*P. humboldti* and *E. gallinacea*)	Colony	Stony Point	Simon's Town	0.090054	0.04393	2.05	*
			Stony Point	Dassen Island	-0.086544	0.049019	-1.766	.
			Stony Point	Dyer Island	-0.314059	0.060882	-5.158	***
			Stony Point	Robben Island	0.030888	0.044782	0.69	.
		Year	Year 2016	Year 2017	0.082524	0.032126	2.569	*
		Age	Adult	Chick	0.431918	0.051295	8.42	***
		Body mass			-0.003335	0.02564	-0.13	.
glm 'binomial'	Lice (*A. demersus*)	Location	Islands	Mainland	-1.2042	0.826	-1.458	.
		Year	Year 2016	Year 2017	1.0575	0.825	1.282	.
		Age	Adult	Chick	17.9121	1997.992	0.009	.
		Body mass			0.7762	0.5929	1.309	.
glm 'binomial'	Lice (*A. demersus*)	Colony	Stony Point	Simon's Town	-16.48937	2429.78679	-0.007	.
			Stony Point	Dassen Island	1.23637	0.93271	1.326	.
			Stony Point	Dyer Island	0.95618	1.02634	0.932	.
			Stony Point	Robben Island	0.03707	1.23663	0.03	.
		Year	Year 2016	Year 2017	1.06924	0.82846	1.291	.
		Age	Adult	Chick	17.85439	1936.21697	0.009	.
		Body mass			0.82471	0.61627	1.338	.

glm 'binomial'	Ticks (*O. capensis* s. s.)	Location	Islands	Mainland	0.4426	0.2845	1.556	.
		Year	Year 2016	Year 2017	0.6829	0.2899	2.355	*
		Age	Adult	Chick	2.2412	0.7432	3.016	**
		Body mass			-0.4595	0.2194	-2.094	*
glm 'binomial'	Ticks (*O. capensis* s. s.)	Colony	Stony Point	Simon's Town				
					-2.436	0.747	-3.261	**
			Stony Point	Dassen Island				
					-1.0322	0.4444	-2.323	*
			Stony Point	Dyer Island				
					-1.527	0.512	-2.982	**
			Stony Point	Robben Island				
					-0.4048	0.3741	-1.082	.
		Year	Year 2016	Year 2017	0.6398	0.2956	2.165	*
		Age	Adult	Chick	2.0568	0.7473	2.752	**
		Body mass			-0.6267	0.2304	-2.72	**
HAEMOPARASITES								
glm 'binomial'	Piroplasmids/Haemosporida	Location	Islands	Mainland	1.9782	0.2148	9.211	***
		Year	Year 2016	Year 2017	-0.1542	0.2028	-0.76	.
		Age	Adult	Chick	2.3298	0.3198	7.286	***
		Body mass			0.396	0.1639	2.415	*
glm 'binomial'	Piroplasmids/Haemosporida	Colony	Stony Point	Simon's Town	-0.5709	0.275	-2.076	*
			Stony Point	Dassen Island	-3.0786	0.4518	-6.815	***
			Stony Point	Dyer Island	-1.2171	0.2873	-4.236	***

			Stony Point	Robben Island	-2.7051	0.3871	-6.987	***
		Year	Year 2016	Year 2017	-0.178	0.2079	-0.856	.
		Age	Adult	Chick	2.3439	0.3273	7.161	***
		Body mass			0.4331	0.169	2.563	**
glm 'binomial'	Spirochaetales	Location	Islands	Mainland	0.3803	0.691	0.55	.
		Year	Year 2016	Year 2017	-0.1871	0.6869	-0.272	.
		Age	Adult	Chick	15.6435	1279.9671	0.012	.
		Body mass			-1.2012	0.5977	-2.01	*
glm 'binomial'	Spirochaetales	Colony	Stony Point	Simon's Town				
					-0.84	1.1558	-0.727	.
			Stony Point	Dassen Island				
					-0.8268	1.1453	-0.722	.
			Stony Point	Dyer Island				
					-0.3314	0.9199	-0.36	.
			Stony Point	Robben Island				
					-0.8765	1.148	-0.764	.
		Year	Year 2016	Year 2017	-0.2631	0.6944	-0.379	.
		Age	Adult	Chick	15.5082	1275.2	0.012	.
		Body mass			-1.2447	0.6139	-2.028	*
HELMINTHS								
glm 'binomial'	*Cardiocephaloides* spp.	Location	Islands	Mainland	0.7119	0.2317	3.073	**
		Year	Year 2016	Year 2017	-1.4495	0.2351	-6.166	***
		Body mass			0.5537	0.1777	3.115	**
glm 'binomial'	*Cardiocephaloides* spp.	Colony	Stony Point	Simon's Town	-0.3159	0.3438	-0.919	.

			Stony Point	Dassen Island	-0.8585	0.3538	-2.426	*
			Stony Point	Dyer Island	-0.7548	0.3519	-2.145	*
			Stony Point	Robben Island	-0.8319	0.3531	-2.356	*
		Year	Year 2016	Year 2017	-1.4688	0.2382	-6.167	***
		Body mass			0.5373	0.1823	2.947	**
glm 'binomial'	*Contracaecum* spp.	Location	Islands	Mainland	0.2581	0.3242	0.796	.
		Year	Year 2016	Year 2017	-0.217	0.3209	-0.676	.
		Body mass			0.1174	0.2388	0.492	.
glm 'binomial'	*Contracaecum* spp.	Colony	Stony Point	Simon's Town				
					0.20077	0.44982	0.446	.
			Stony Point	Dassen Island				
					-0.92081	0.64555	-1.426	.
			Stony Point	Dyer Island				
					-0.01606	0.48786	-0.033	.
			Stony Point	Robben Island				
					0.10546	0.45703	0.231	.
		Year	Year 2016	Year 2017	-0.1686	0.3234	-0.521	.
		Body mass			0.14301	0.24712	0.579	.
glm 'binomial'	*Cyathostoma* spp.	Location	Islands	Mainland	1.2018	0.8048	1.493	.
		Year	Year 2016	Year 2017	-17.7061	1333.4277	-0.013	.
		Body mass			0.8867	0.4961	1.787	.
glm 'binomial'	*Cyathostoma* spp.	Colony	Stony Point	Simon's Town				
					-18.4971	3542.6623	-0.005	.

			Stony Point	Dassen Island	-18.3539	3563.03	-0.005	.
			Stony Point	Dyer Island	-0.2418	0.918	-0.263	.
			Stony Point	Robben Island	-18.8147	3631.9956	-0.005	.
		Year	Year 2016	Year 2017	-18.6116	2068.8749	-0.009	.
		Body mass			0.7407	0.5607	1.321	.
ECTOPARASITES IN NESTS								
ZINB	Total Ectoparasites	Location	Islands	Mainland	0.15051	0.055	2.737	**
		Year	Year 2016	Year 2017	0.21289	0.05421	3.927	***
ZINB	Total Ectoparasites	Colony	Stony Point	Simon's Town	0.21364	0.07566	2.824	**
			Stony Point	Dassen Island	-0.04282	0.08018	-0.534	.
			Stony Point	Dyer Island	-0.1319	0.08966	-1.471	.
			Stony Point	Robben Island	-0.10131	0.08198	-1.236	.
		Year	Year 2016	Year 2017	0.22431	0.05424	4.136	***
ZINB	Total fleas (*P. humboldti* and *E. gallinacea*)	Location	Islands	Mainland	0.16791	0.06119	2.744	**
		Year	Year 2016	Year 2017	0.15607	0.06157	2.535	*
ZINB	Total fleas (*P. humboldti* and *E. gallinacea*)	Colony	Stony Point	Simon's Town	0.23235	0.08304	2.798	**

			Stony Point	Dassen Island	-0.10029	0.08587	-1.168	.
			Stony Point	Dyer Island	-0.03714	0.11402	-0.326	.
			Stony Point	Robben Island	-0.10265	0.08833	-1.162	.
			Year 2016	Year 2017	0.17289	0.06185	2.795	**
ZINB	Adult fleas (*P. humboldti* and *E. gallinacea*)	Location	Islands	Mainland	0.159537	0.074776	2.134	*
		Year	Year 2016	Year 2017	0.006149	0.073633	0.084	.
ZINB	Adult fleas (*P. humboldti* and *E. gallinacea*)	Colony	Stony Point	Simon's Town	0.39834	0.08065	4.939	***
			Stony Point	Dassen Island	0.07608	0.11177	0.681	.
			Stony Point	Dyer Island	0.1021	0.12505	0.816	.
			Stony Point	Robben Island	-0.05592	0.11001	-0.508	.
		Year	Year 2016	Year 2017	0.04379	0.07128	0.614	.
ZINB	Flea larvae (*P. humboldti* and *E. gallinacea*)	Location	Islands	Mainland	0.18582	0.06615	2.809	**
		Year	Year 2016	Year 2017	0.13789	0.06697	2.059	*
ZINB	Flea larvae (*P. humboldti* and *E. gallinacea*)	Colony	Stony Point	Simon's Town	0.21678	0.08927	2.428	*
			Stony Point	Dassen Island	-0.1756	0.09105	-1.929	.

			Stony Point	Dyer Island	0.0246	0.13275	0.185	.
			Stony Point	Robben Island	-0.10019	0.0957	-1.047	.
		Year	Year 2016	Year 2017	0.14373	0.06776	2.121	*
ZINB	Ticks (*O. capensis* s. s.)	Location	Islands	Mainland	0.12994	0.0826	1.573	.
		Year	Year 2016	Year 2017	0.09864	0.0731	1.349	.
ZINB	Ticks (*O. capensis* s. s.)	Colony	Stony Point	Simon's Town	-0.22204	0.09968	-2.228	*
			Stony Point	Dassen Island	-0.4778	0.14262	-3.35	***
			Stony Point	Dyer Island	0.01278	0.10445	0.122	.
			Stony Point	Robben Island	-0.26925	0.15759	-1.708	.
		Year	Year 2016	Year 2017	0.12835	0.07204	1.782	.

Discussion

Parasite diversity and abundance associated with penguins and their nests

In this study, *P. humboldti* was the most prevalent and abundant parasite on penguins and in their nests. Most of the *P. humboldti* found on penguins were adults, while the larval stage dominated the nests. This is consistent with the life cycle of fleas, given that adult fleas mainly attach to the host for a blood meal while the larvae remain in the nest where they feed on organic matter (Boyd, 1951; Bitam *et al.* 2010). The sex ratio of *P. humboldti* was equal on penguins and female-biased in nests (0.7:1). This pattern has previously been observed in nests of other bird (e.g. passerines) and is consistent with the fact that female fleas live longer than males in natural populations and are thus more prevalent (Rothschild and Clay, 1952; Shutler *et al.* 2003). Fleas from the genus *Parapsyllus* are known ectoparasites of penguins (Clarke and Kerry, 1993). Some examples are *P. longicornis* that has been recorded from several species of penguins such as from little penguin (*Eudyptula minor*) and southern rockhopper penguin (*Eudyptes chrysocome*) (Murray *et al.* 1990). Also, *P. magellanicus* and *P. heardi* have been recorded from southern rockhopper penguin (Murray and Vestjens, 1967; Murray *et al.* 1990), and *P. jacksoni* from little penguins (Murray *et al.* 1990). In particular, the flea *P. humboldti* has been found on Humboldt penguins (*Spheniscus humboldti*) and in their nests in Chile and Peru (Jordan, 1942; Segerman, 1995; Smith *et al.* 2008). *Parapsyllus humboldti* is also frequently collected from African penguins in rehabilitation centres in South Africa (Parsons and Vanstreels, 2016), and is currently the only species from the genus *Parapsyllus* in southern Africa (Segerman, 1995).

A species of flea not previously reported for African penguins, the sticktight flea (*E. gallinacea*) was attached to the eyelids and body of penguins and recorded in the nests at Dassen Island. *Echidnophaga gallinacea* has a worldwide-distribution (Boughton *et al.* 2006) and although it is a known flea of rodents, it infests an extensive variety of hosts, including poultry, domestic mammals and wildlife (Boyd, 1951; Segerman, 1995; Gyimesi *et al.* 2007; Bitam *et al.* 2010). In South Africa, *E. gallinacea* has been collected from dogs, cats, rats and fowls (Waterston, 1914), and from wild carnivores (e.g. black-footed cat *Felis nigripes* and Cape fox *Vulpes chama*) (Horak *et al.* 2004; Matthee *et al.* 2011). Female fleas remain firmly attached on the host for long periods and have a high fecundity (Horak *et al.* 2004; Krasnov, 2008). In the present study, *E. gallinacea* recorded the second highest mean intensity on penguins (4.95±0.60). The presence of this flea on Dassen Island may have been facilitated by the presence of the European rabbit (*Oryctolagus cuniculus*) as the flea has been reported from the burrows of this rabbit species in other parts of the world (Dunnet and Nardon, 1974). Outside South Africa, flea infestation by members of Pulicidae have been detected in seabirds such as

Scopoli's shearwater (*Calonectris diomedea diomedea*), Atlantic Cory's shearwater (*Calonectris diomedea borealis*) and Cape Verde shearwater (*Calonectris edwardsii*) (Guiguen *et al.* 1989; Gómez-Díaz *et al.* 2008). *Echidnophaga gallinacea* showed a female-biased ratio on penguins (0.16:1); something expected since the female of this flea species attaches to the hosts for long periods of time (Boughton *et al.* 2006).

The chewing louse *A. demersus* was the only louse species found on penguins in the study. Although there are only a few reports on lice of African penguins, this species is considered a typical parasite of African penguins (Von Keler, 1952; Banks and Palma, 2003). *Austrogoniodes demersus* has also been sporadically recorded from Galápagos penguins (*Spheniscus mendiculus*) in the Galápagos Archipelago (Banks and Palma, 2003). The genus *Austrogoniodes* seems to be associated with multiple penguin species (Pilgrim and Palma, 1982; Clarke and Kerry, 1993) with 15 *Austrogoniodes* species infesting penguins (Clay, 1967; Banks and Palma, 2003) and 1 species (*A. metoecus*) infests ducks (musk duck *Biziura lobata*) (Clay, 1971). The on-host sex ratio for *A. demersus* was female-biased (0.38:1). The predominant presence of female lice on birds has been previously reported, and might be the result of the larger female longevity and the smaller size and active lifestyle of males (Marshall, 1981*b*; Clayton *et al.* 1992; Sychra *et al.* 2011; Pap *et al.* 2013). Lice transmission occurs by direct body contact between individuals such as between parents to offspring in the nest (Clayton and Tompkins, 1994, 1995) and between older chicks when they group together in the crèche stage (Banks *et al.* 2006; Rivera-Parra *et al.* 2014).

The soft tick *O. capensis* sensu stricto (s. s.) is a broadly recognized ectoparasite of marine birds (Hoogstraal *et al.* 1985). It belongs to the *O. capensis* sensu lato (s. l.) group, which comprises 11 morphologically associated species (Muñoz-Leal *et al.* 2017). The *O. capensis* (s. l.) group is widely distributed in mainland and island colonies in tropical and temperate regions across the world (Hoogstraal *et al.* 1985; Keirans *et al.* 1992). In particular, *O. capensis* (s. s.) infests several seabird species globally (e.g. Hoogstraal *et al.* 1976; Keirans *et al.* 1992; Dupraz *et al.* 2016). In South Africa, *O. capensis* (s. s.) has been collected from Cape cormorant (*Phalacrocorax capensis*) (Peirce and Parsons, 2012), great black-backed gull (*Larus marinus*), kelp gull (*Larus dominicanus*), Cape gannet (*Morus capensis*) (Theiler, 1959) and African penguins (Theiler, 1959; Hoogstraal *et al.* 1985; Daturi, 1986; Duffy and Daturi, 1987). *Ornithodoros capensis* (s. s.) is a nidicolous soft tick species (Sonenshine, 1993). After a blood meal, the females lay several small batches of eggs (up to 500/cycle) from where larvae emerge to then become nymphs. Soft ticks go through numerous nymphal instars (up to 8) and the resulting adult tick is then ready to mate off the host and is able to live in the host's shelter for long periods of time (with a maximum life span of 25 years) (Sonenshine, 1991, 1993; Vial,

2009). Each life stage generally attaches to the host for short blood meals (from a few minutes up to an hour) and while immature stages usually feed only once adult ticks feed several times (Oliver, 1989). The colonial lifestyle, permanent use of the same nest sites in successive years and the high population densities that penguins reach, expose them to a greater abundance of ticks (Duffy, 1988; Mangin *et al.* 2003). In our study, *O. capensis* (s. s.) was the second most abundant ectoparasites found on penguins. Although larvae from some species within the genus *Ornithodoros* do not take blood meals and, therefore, do not occur on the host (e.g. *O. moubata*; Vial, 2009), we confirmed that the larval stage of *O. capensis* (s. s.) does feed on penguins due to the presence of larvae on African penguins at all five colonies and the presence of blood in the intestines of the larvae. This life stage was also the most abundant on penguins in the majority of the selected colonies. In nests, *O. capensis* (s. s.) was also the second most prevalent and abundant parasite, and exhibited one of the highest mean intensity of infestation. Nymphs were the most prevalent and abundant life stage in all nests, while larvae recorded the highest mean intensity in nests. This agrees with the findings of Daturi (1986) that recorded a higher abundance of larvae than adult ticks when collecting *O. capensis* from nests of African penguins at Marcus Island. In the present study, only female ticks were recorded on penguins, while in nests the tick showed a strong bias towards males. In many nidicolous tick species, males require fewer nymphal stages to emerge as adults (i.e. become adults sooner) compared to females (Sonenshine, 1991). This could explain the presence of more male than female ticks in penguin nests. In fact, it is not unusual to find large numbers of male nidicolous ticks in the host nests (e.g. *Argas arboreus* in nests of cattle egrets (*Bubulcus ibis*); Guirgis, 1971).

In this study Piroplasmorida/Haemospororida (orders that include *Babesia* spp., *Plasmodium* spp. and *Leucocytozoon* spp.; Levine, 1971; Atkinson, 2008) were more commonly recorded in penguins (33.51%) compared to Spirochaetales (2.59%) (order that includes *Borrelia* spp.; Paster *et al.* 1991). It is possible that Piroplasmorida/Haemospororida are more prevalent in penguin species compared to Spirochaetales. A recent study by Quillfeldt *et al.* (2011) recorded an average prevalence of 14.4% for Piroplasmorida/Haemospororida in 19 penguin species (including African penguins) across different geographic areas. While a study on African penguins recorded a lower prevalence (1.4%) for *Borrelia* spp. in South Africa (Yabsley *et al.* 2012). Haemoparasites within the taxonomic orders identified in the present study have previously been recorded in several penguin species (Jones and Shellam, 1999). The blood pathogens detected in this study are transmitted by hematophagous arthropods, including mosquitoes (Culicidae), black flies (Simuliidae) and soft ticks (*Ornithodoros* spp.) (Atkinson and van Riper III, 1991; Yabsley *et al.* 2012). Although hard ticks are recognized vectors of *Babesia* spp. (Earlé *et al.* 1993), we believe that the high prevalence of the *Babesia*-like

inclusions in erythrocyte observed in this study is related to *O. capensis* as it was the only tick species detected on penguins and in their nests.

Helminth species from four genera (*Cardiocephaloides*, *Renicola*, *Contracaecum* and *Cyathostoma*) were recorded from penguin chicks. Previously, the trematodes *Cardiocephaloides physalis* and *Renicola sloanei*, and the nematodes *Contracaecum* sp., *Contracaecum variegatum* and *Cyathostoma phenisci* were recorded from African penguins (Randall and Bray, 1983; Fagerholm *et al.* 1996; Horne *et al.* 2011; Kanarek *et al.* 2013; Viljoen, 2015; Parsons and Vanstreels, 2016; Vanstreels, 2016). Most of these helminths have been associated with various penguin species, which may be related to their similarity in diet (Brandão *et al.* 2014). *Cardiocephaloides* spp. was the most prevalent (56.17%) helminth genus recorded in the study followed by *Contracaecum* spp. (12.83%) and *Cyathostoma* spp. (3.87%). These results are similar to those found in African penguins (of all ages) admitted for rehabilitation in the Western Cape Province (Viljoen, 2015). Nematodes, trematodes, cestodes and acanthocephalans have been reported from different penguin species, where they infect gastrointestinal tract, liver, bile duct, kidney and trachea (Clarke and Kerry, 1993; Duignan, 2001). They infect both male and female penguins, but studies suggest a higher prevalence in younger birds (Clarke and Kerry, 1993; Viljoen, 2015). Since the life cycle of the helminth parasites involves fish, squid and krill, it is likely that penguins acquired infection through their diet (Randall and Bray, 1983; Horne *et al.* 2011; Brandão *et al.* 2014). The integrity of the immune system, type of diet and behaviour of penguins will however determine the degree of susceptibility to helminth infections (Diaz, 2006; Diaz *et al.* 2010; Carrera-Játiva *et al.* 2014).

Factors that influence parasite infestations

Significantly more ectoparasites, and particularly fleas (*P. humboldti* and *E. gallinacea*) and ticks (*O. capensis* s. s.) were recorded on chicks compared to adult penguins. Chicks generally have a less developed immune system and are therefore more susceptible to parasitic infestations compared to adult penguins (van Rensburg, 2010; Yabsley *et al.* 2012). In addition, chicks spend more time in or close to the nest (Sherley *et al.* 2014) and are therefore more readily infested by nest-associated ectoparasites (fleas and soft ticks). Since ticks can act as vector of haemoparasites, the significantly higher incidence of Piroplasmorida/Haemospororida in chicks compared to adult penguins, in the present study, could potentially be a reflection of the pattern observed for *O. capensis* (s. s.) (Peirce, 2000).

Parasite infestations were significantly higher in mainland compared to island colonies. Stony Point and Simon's Town, the two mainland colonies, exhibited the same parasite richness but higher abundance and prevalence of parasites on and in penguins and in their nests than on

islands. Although some studies suggest that parasites develop an "island syndrome" (Nieberding *et al.* 2006), where parasites on islands show lower species richness, lower host-specificity, and higher abundance and prevalence compared to those on mainland (Fromont *et al.* 2001; Literák *et al.* 2015), there are also studies that have found a decrease in parasite prevalence on islands compared to continental parasites (Pérez-Rodríguez *et al.* 2013). In this study, the most likely explanation for this pattern is the higher densities of both total and active nests, on mainland compared to island colonies. High nest density in seabird colonies is a recognized factor that influences ectoparasite abundance (Duffy, 1983; Duffy, 1988; Ramos and Drummond, 2017). Living in large colonies with nests at close proximity allows proliferation and transmission of ectoparasites (Brown and Brown, 1986, 2004) with a subsequent potential impact on animal health and condition (Lehmann, 1993). Some parasites, such as nidicolous soft ticks, are able to remain in nests regardless of the presence of a bird in the nest (Duffy, 1983; Duffy, 1988). While other parasites, such as fleas, persist and are abundant only when the nest is active (Marshall, 1981*a*). This was supported by our results, which showed a positive correlation between mean abundance of *O. capensis* (s. s.) in nests and total nest density (active and non-active together), while mean total ectoparasite abundance in nests (of which fleas represented 71.57%) correlated with the density of active nests. The positive correlation between total nest density and Piroplasmids/Haemospororida prevalence in penguins is most probably due to a higher abundance of *O. capensis* (s. s.) in colonies with higher nests densities. Coloniality in birds seems to facilitate elevated haemoparasite richness and prevalence (Tella, 2002). From the present study it appears that higher total nest density can further aggravate the situation.

Only one (*Cardiocephaloides* spp.) of the four helminth genera was significantly more prevalent in chicks at the two mainland colonies compared to islands. Species in the genus *Cardiocephaloides* require sea snails (first intermediate host), fish (second intermediate hosts) and seabirds, such as penguins, (definitive host) to complete their life cycle (Born-Torrijos *et al.* 2016). The complete life cycle of *C. physalis*, the most likely *Cardiocephaloides* species found in our study, has not been fully described but it is hypothesized that it uses a *Burnupena* spp. as its first intermediate host (Ukomadu, 2017). The presence of natural predators of *Burnupena* spp. (e.g. rock lobster) can influence its spatial distribution (Caro, 2010), and therefore the availability of a suitable intermediate host for *Cardiocephaloides* spp. (Born-Torrijos *et al.* 2016). The South Coast rock lobster (*Palinurus gilchristi*) is commercially fished between the west coast (Cape Point) to the southeast coast (East London) of South Africa (Fig. 2.1). This range includes the two mainland colonies (Stony Point and Simon's Town) and Dyer Island (Department of Environmental Affairs, 2013). Although Stony Point is located in

a marine protected area, it is also near the fishing zone of the West Coast rock lobster (*Jasus lalandii*) (Blamey *et al*. 2013). This could affect the abundance of rock lobster in the area and facilitate a larger abundance of *Burnupena* spp. The presence of the second intermediate host is also important and acoustic surveys recorded higher densities of sardines (food source of penguins and second intermediate host of *Cardiocephaloides* spp.) along the south-western coast between Cape Point and Cape Agulhas (Grémillet *et al.* 2008; Reed *et al.* 2012; Mhlongo *et al.* 2013). Moreover, studies have recorded higher infestation of *Cardiocephaloides* spp. in sardines off the west coast and specifically between Cape Point and Cape Agulhas compared to the west coast north of Cape Point and the south coast (Van der Lingen *et al.* 2015; Weston *et al.* 2015). From this, it is possible that the presence of intermediate and definitive hosts facilitates a higher incidence of *Cardiocephaloides* spp. in penguins at the two mainland colonies. Although Dyer Island falls within the abovementioned range, it is possible that the inaccessibility of the island to humans may facilitate a healthier lobster population in the immediate area around the island. This would explain why the incidence of *Cardiocephaloides* spp. in penguin chicks was lower compared to the two mainland colonies.

When assessing the effect of colony on the parasite infestation in penguins it is evident that there must be certain conditions that facilitate higher on-host infestations of *O. capensis* (s. s.) at Stony Point compared to the other colonies. Higher on-host abundances of *O. capensis* (s. s.) is possibly due to the significantly higher abundance of *O. capensis* (s. s.) in nests, which would explain the higher incidence of Piroplasmids/Haemospororida in penguins from Stony Point. This pattern may be related to a consistent annual increase in the penguin population at Stony Point (466 breeding pairs in 2010 to 2388 in 2016), while the size of the other colonies either remained approximately constant (e.g. Simon's Town: 933 to 870 pairs, respectively) or decreased in numbers (e.g. Dassen Island decreases from 4929 to 1862 pairs, respectively) (CapeNature, DEA and SANParks unpublished results). This influx of penguins may explain the higher total nest density (0.28 nests/m^2) and *O. capensis* (s. s.) abundance on penguins and in nests at Stony Point compared to the other colonies. Interestingly, the Simon's Town colony had the second highest total nest density, but a higher active nest density (0.14 nests/m^2), although only slightly, compared to Stony Point (0.13 nests/m^2) (Table 2.1). Given the close association between fleas and nest occupancy (Marshall, 1981*a*) it is not surprising that total ectoparasite abundance (of which fleas represented >70%), total fleas, adult fleas and flea larvae were higher in active nests at Simon's Town compared to the other colonies.

Very limited deductions can be made from the relationship between the remote sensed climate data and parasite infestation. It appears that there is a significant negative relationship between annual mean temperature and annual precipitation and *A. demersus* infestation on

penguins sampled at different colonies. According to Johnson and Clayton (2003), chewing lice are severely affected by temperature and humidity near the host skin, and an experimental study did record a negative relationship between lice survival and high ambient temperature (Chen and Mullens, 2008). Conversely, there was a positive correlation between annual mean temperature and annual precipitation and prevalence of *Contracaecum* spp. in penguin chicks. Dassen Island recorded the lowest annual mean temperature and annual precipitation compared to the other colonies and the prevalence of *Contracaecum* spp. was the lowest at this colony. Interestingly, the sea surface temperature around Dassen Island also seems to be the coldest compared to the other colonies (Grémillet *et al.* 2008). Sea water temperature is one of the factors that can impact the distribution of pelagic fish (Sabatés *et al.* 2006) and studies have recorded an east-ward shift, towards Cape Agulhas, in fish distribution (Grémillet *et al.* 2008). Likewise, hatching time of Anisakidae larva (helminth family of *Contracaecum* spp.) is affected by water temperature and tends to be delayed at colder temperatures (Højgaard, 1998). This might affect the infection rate of intermediate hosts, which may result in a lower prevalence of *Contracaecum* spp. in penguins (one of the definitive hosts). However, these arguments are speculative and will need to be confirmed in future studies.

Seasonal variation in parasite infestation

Parapsyllus humboldti and *O. capensis* (s. s.) prevalence and abundance on penguin chicks and in nests were higher in spring compared to the colder and wet autumn/winter. Possible drivers of this pattern may be the absence of caring adults (e.g. providing allopreening and food) for the chicks and more favourable climatic conditions for parasites during spring. In South Africa, the moult stage of the African penguin takes place during spring and summer (September and January) (Crawford *et al.* 2006). During the moulting period, adult penguins fast for about 21 days leaving the penguin chicks unattended in the nests (Sherley *et al.* 2014). This can have a significant effect on the parasite infestation of chicks as allopreening between birds (a natural behaviour exhibited by adult penguins on chicks; Brooke, 1985) have been shown to reduce ectoparasite infestations (Brooke, 1985; Villa *et al.* 2016). Chicks are also dependent on adults for food (Williams and Cooper, 1984) and this, together with a higher parasite infestation, can reduce the body condition of chicks during spring (Obendorf and McColl, 1980; Roulin *et al.* 2003). In addition, the colonial behaviour of penguins facilitates a build-up of ectoparasites and especially soft ticks in nests and in the colony. It is possible that the abundance of *O. capensis* (s. s.) increased during the first breeding season and thereafter were retained in the nests and colony. This, together with more favourable climatic conditions for tick development (warmer

and drier climatic conditions) could facilitate higher infestation of *O. capensis* (s. s.) in nests in spring (Lees, 1947).

Spirochaetales were the only haemoparasites group that exhibited a significant increase in penguins during spring compared to autumn/winter. Similar results have been documented by Yabsley *et al.* (2012), who found a higher prevalence of *Borrelia* spp. in blood smears from African penguins sampled during spring-summer months (October to February) compared to autumn-winter months (March to September) in South Africa. It is possible that seasonal changes in tick activity can influence seasonal changes in the prevalence of *Borrelia* spp. (Furuno *et al.* 2017). In this study, the higher prevalence of Spirochaetales in the warmer season coincides with the higher abundance and prevalence of *O. capensis* (s. s.) infesting penguins in the spring compared to autumn/winter.

The present study provides current information on the parasite diversity of natural occurring African penguins and their nests at colonies along the south-western coast of South Africa. In general, penguin chicks are more susceptible to parasite infestations during spring. Further, it is evident that the observed spatial variation in parasite infestations between colonies is driven by several factors. In particular, patterns recorded for ecto- and haemoparasites tend to be facilitated by nest density, while the availability of infected prey influences helminth infestations. Knowing and detecting changes in parasitic diversity and abundance can give insight into the possible intrinsic and extrinsic factors that may threaten the conservation of African penguins in the region.

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Supplementary material

Table S2.1. Ectoparasites, haemoparasites and helminths obtained from African penguins at five colonies along the south-western coast of South Africa during autumn/winter 2016 and 2017. Sample sizes N=690 (ectoparasites), 640 (haemoparasites) and 363 (helminths).

Parasite species	Colony	Total prevalence (%)	Mean abundance (±SE)				Mean intensity (±SE)				Sex:ratio (♂:♀)
			Larvae	Nymphs	Adults	Total	Larvae	Nymphs	Adults	Total	
Fleas											
P. humboldti	Dassen Island	40.83[1]	-	-	1.77 (±0.30)	1.77 (±0.30)[1]	-	-	4.33 (±0.56)	4.33 (±0.56)[1]	0.87:1
	Dyer Island	44.17	0	-	1.6 (±0.27)	1.6 (±0.27)	0	-	3.62 (±0.49)	3.62 (±0.49)	1.46:1
	Robben Island	75.83	0	-	5.18 (±0.57)	5.18 (±0.57)	0	-	6.84 (±0.67)	6.84 (±0.67)	1.22:1
	Stony Point	78.57	0	-	5.02 (±0.34)	5.02 (±0.34)	0	-	6.39 (±0.37)	6.39 (±0.37)	1.11:1
	Simon's Town	80	0	-	6.19 (±0.59)	6.19 (±0.59)	0	-	7.74 (±0.64)	7.74 (±0.64)	0.93:1
E. gallinacea	Dassen Island	35	-	-	1.73 (±0.30)	1.73 (±0.30)	-	-	4.95 (±0.60)	4.95 (±0.60)	0.16:1
Lice											
A. demersus	Dassen Island	2.50	-	-	0.05 (±0.04)	0.05 (±0.04)	-	-	2 (±1)	2 (±1)	1:1
	Dyer Island	1.67	-	-	0.02 (±0.01)	0.02 (±0.01)	-	-	1	1	only female

	Robben Island	0.83	-	-	0.01 (±0.01)	0.01 (±0.01)	-	-	1	1	only female
	Stony Point	0.95	-	-	0.01 (±0.01)	0.01 (±0.01)	-	-	1	1	only female
	Simon's Town	0	-	-	0	0	-	-	0	0	0
Ticks											
O. capensis	Dassen Island	5.83	0.03 (±0.02)	0.22 (±0.18)	0	0.25 (±0.18)	1.33 (±0.33)	6.5 (±5.17)	0	4.29 (±2.96)	0
	Dyer Island	4.17	0.1 (±0.05)	0.02 (±0.01)	0	0.12 (±0.06)	2.40 (±0.68)	1	0	2.80 (±0.80)	0
	Robben Island	10	0.13 (±0.04)	0.01 (±0.01)	0	0.13 (±0.04)	1.36 (±0.20)	1	0	1.33 (±0.19)	0
	Stony Point	15.71	0.31 (±0.08)	0.03 (±0.01)	0.005 (±0.005)	0.35 (±0.08)	2.28 (±0.39)	1	1	2.21 (±0.35)	only female
	Simon's Town	1.67	0.01 (±0.01)	0.01 (±0.01)	0	0.02 (±0.01)	1	1	0	1	0
Haemoparasites[2]											
Piroplasmorida/ Haemosporida	Dassen Island	5.31	-	-	-	-	-	-	-	-	-
	Dyer Island	23.58	-	-	-	-	-	-	-	-	-
	Robben Island	7.83	-	-	-	-	-	-	-	-	-
	Stony Point	55.61	-	-	-	-	-	-	-	-	-
	Simon's Town	35.64	-	-	-	-	-	-	-	-	-

Spirochaetales	Dassen Island	0.88	-	-	-	-	-	-	-	-	-	
	Dyer Island	1.89	-	-	-	-	-	-	-	-	-	
	Robben Island	0.87	-	-	-	-	-	-	-	-	-	
	Stony Point	1.95	-	-	-	-	-	-	-	-	-	
	Simon's Town	0.99	-	-	-	-	-	-	-	-	-	
Helminths[2]												
Cardiocephaloides spp.	Dassen Island	41.51	-	-	-	-	-	-	-	-	-	
	Dyer Island	46.43	-	-	-	-	-	-	-	-	-	
	Robben Island	48.15	-	-	-	-	-	-	-	-	-	
	Stony Point	63.38	-	-	-	-	-	-	-	-	-	
	Simon's Town	56.90	-	-	-	-	-	-	-	-	-	
Renicola spp.	Dassen Island	0	-	-	-	-	-	-	-	-	-	
	Dyer Island	0	-	-	-	-	-	-	-	-	-	
	Robben Island	0	-	-	-	-	-	-	-	-	-	
	Stony Point	0	-	-	-	-	-	-	-	-	-	
	Simon's Town	0	-	-	-	-	-	-	-	-	-	
Contracaecum spp.	Dassen Island	5.66	-	-	-	-	-	-	-	-	-	
	Dyer Island	12.5	-	-	-	-	-	-	-	-	-	
	Robben Island	14.81	-	-	-	-	-	-	-	-	-	
	Stony Point	13.38	-	-	-	-	-	-	-	-	-	
	Simon's Town	15.52	-	-	-	-	-	-	-	-	-	
Cyathostoma spp.	Dassen Island	0	-	-	-	-	-	-	-	-	-	
	Dyer Island	3.57	-	-	-	-	-	-	-	-	-	
	Robben Island	0	-	-	-	-	-	-	-	-	-	
	Stony Point	6.34	-	-	-	-	-	-	-	-	-	
	Simon's Town	0	-	-	-	-	-	-	-	-	-	

[1]Total prevalence, mean abundance and mean intensity of fleas in Dassen Island excluded larvae.

[2]Prevalence of haemo- and endoparasites is the presence of parasites at each group or genus from the total of hosts examined at each colony.

Table S2.2. Ectoparasites obtained from nests of African penguins (N=547) in five colonies along the south-western coast of South Africa during autumn/winter 2016 and 2017.

Parasite species	Colony	Prevalence (%)				Mean abundance (±SE)				Mean intensity (±SE)				Sex:ratio (♂:♀)
		Larvae	Nymphs	Adults	Total	Larvae	Nymphs	Adults	Total	Larvae	Nymphs	Adults	Total	
Fleas														
P. humboldti	Dassen Island[1]	-	-	25	25	-	-	0.80 (±0.35)	0.80 (±0.35)	-	-	3.2 (±1.28)	3.2 (±1.28)	0.49:1
	Dyer Island	26.25	-	22.50	36.25	8.25 (±2.68)	-	0.63 (±0.20)	8.88 (±2.69)	31.43 (±8.48)	-	2.78 (±0.67)	24.48 (±6.54)	0.53:1
	Robben Island	61.25	-	32.50	70	10.49 (±2.18)	-	0.76 (±0.26)	11.25 (±2.20)	17.12 (±3.23)	-	2.35 (±0.73)	16.07 (±2.92)	0.43:1
	Stony Point	56.39	-	29.96	62.11	15.51 (±2.17)	-	0.73 (±0.12)	16.24 (±2.22)	27.51 (±3.50)	-	2.43 (±0.32)	26.14 (±3.30)	0.83:1
	Simon's Town	70	-	61.25	77.50	39.30 (±8.62)	-	3.14 (±0.48)	42.44 (±8.97)	56.14 (±11.63)	-	5.12 (±0.63)	54.76 (±11.10)	0.76:1
E. gallinacea	Dassen Island	-	-	5	5	-	-	0.08 (±0.04)	0.08 (±0.04)	-	-	1.5 (±0.29)	1.5 (±0.29)	1:1
Lice														
A. demersus	Dassen Island	-	-	1.25	1.25	-	-	0.01 (±0.01)	0.01 (±0.01)	-	-	1	1	only female
	Dyer Island	-	-	0	0	-	-	0	0	-	-	0	0	0

	Robben Island	-	-	0	0	-	-	0	0	-	-	0	0	0
	Stony Point	-	-	0	0	-	-	0	0	-	-	0	0	0
	Simon's Town	-	-	0	0	-	-	0	0	-	-	0	0	0
Ticks														
O. capensis	Dassen Island	11.25	17.50	2.50	26.25	0.11 (±0.04)	0.36 (±0.14)	0.03 (±0.02)	0.50 (±0.15)	1	2.07 (±0.62)	1	1.90 (±0.45)	only male
	Dyer Island	12.50	33.75	27.50	46.25	0.38 (±0.17)	2.10 (±0.78)	1.06 (±0.34)	3.54 (±1.20)	3 (±1.09)	6.22 (±2.13)	3.86 (±1.03)	7.65 (±2.43)	0.51:1
	Robben Island	2.5	16.25	6.25	18.75	0.13 (±0.10)	0.35 (±0.12)	0.13 (±0.07)	0.60 (±0.25)	5 (±3)	2.15 (±0.53)	2 (±0.63)	3.2 (±1.12)	0.67:1
	Stony Point	22.47	47.58	44.93	66.08	2.32 (±1.00)	4.44 (±2.56)	3.70 (±1.79)	10.47 (±4.90)	10.33 (±4.29)	9.33 (±5.35)	8.25 (±3.95)	15.84 (±7.38)	1.93:1
	Simon's Town	21.25	36.25	20	51.25	0.25 (±0.06)	1.01 (±0.27)	0.33 (±0.10)	1.59 (±0.31)	1.18 (±0.13)	2.79 (±0.62)	1.73 (±0.33)	3.10 (±0.50)	1.11:1

[1]Prevalence, mean abundance and mean intensity of flea larvae are excluded in Dassen Island.

Table S2.3. Model selection based on Akaike Information Criterion (AIC). Numbers in brackets represent the different scenarios derived from the full model (assessed in the study) to reach the best model (in bold). Significant differences between the full and the best model (p-value) according to Chi-square test. Independent variables: location (mainland and island); colony (Stony Point, Simon's Town, Dassen-, Dyer- and Robben Island), year (2016 and 2017), penguin age (adult and chick) and body mass.

Model	AIC	Chisq	df	p-value
ON-HOST ECTOPARASITES				
Y=Total on-host ectoparasites				
(1) Total on-host ectoparasites ~ location + year + age + body mass	3286.88			
(2) Total on-host ectoparasites ~ location + year + age	3283.78	3.1	1	>0.05
(1) Total on-host ectoparasites ~ colony + year + age + body mass	3233.06			
(2) Total on-host ectoparasites ~ colony + year + age	3231.14	1.92	1	>0.05
Y=On-host fleas				
(1) on-host fleas ~ location + year + age + body mass	3258.64			
(2) on-host fleas ~ location + year + age	3255.7	2.94	1	>0.05
(1) on-host fleas ~ colony + year + age + body mass	3203.32			
(2) on-host fleas ~ colony + year + age	3201.17	2.15	1	>0.05
Y=Lice				
(1) lice ~ location + year + age + body mass	85.34			
(2) lice ~ location + year + age	85.18	0.16	1	>0.05
(1) lice ~ colony + year + age + body mass	88.62			
(2) lice ~ year + age + body mass	85.81			
(3) lice ~ year + age	85.48	3.14	2	>0.05
Y=On-host ticks				
(1) on-host ticks ~ location + year + age + body mass	371.76	0	0	NA
(1) on-host ticks ~ colony + year + age + body mass	353.89	0	0	NA

HAEMOPARASITES				
Y=Piroplasmids/Haemosporida				
(1) Piroplasmids/Haemosporida ~ location + year + age + body mass	604.42			
(2) Piroplasmids/Haemosporida ~ location + age + body mass	603	1.42	1	>0.05
(1) Piroplasmids/Haemosporida ~ colony + year + age + body mass	583.97			
(2) Piroplasmids/Haemosporida ~ colony + age + body mass	582.71	1.26	1	>0.05
Y=Spirochaetales				
(1) Spirochaetales ~ location + year + age + body mass	93.21			
(2) Spirochaetales ~ location + age + body mass	91.29			
(3) Spirochaetales ~ age + body mass	89.58	3.63	2	>0.05
(1) Spirochaetales ~ colony + year + age + body mass	98.34			
(2) Spirochaetales ~ year + age + body mass	91.52			
(3) Spirochaetales ~ age + body mass	89.58	8.76	2	<0.05
HELMINTH PARASITES				
Y=Cardiocephaloides spp.				
(1) Cardiocephaloides ~ location + year + body mass	448.94	0	0	NA
(1) Cardiocephaloides ~ colony + year + body mass	454.02	0	0	NA
Y=Contracaecum spp.				
(1) Contracaecum ~ location + year + body mass	282.04			
(2) Contracaecum ~ location + year	280.28			
(3) Contracaecum ~ location	278.7			
(4) Contracaecum ~ 1	277.41	4.63	3	>0.05
(1) Contracaecum ~ colony + year + body mass	285.25			
(2) Contracaecum ~ year + body mass	280.68			
(3) Contracaecum ~ year	279			
(4) Contracaecum ~ 1	277.41	7.84	3	<0.05

Y=Cyathostoma spp.				
(1) Cyathostoma ~ location + year + body mass	85.38	0	0	
(1) Cyathostoma ~ colony + year + body mass	81.22			
(2) Cyathostoma ~ colony + year	81.08	0.14	1	>0.05
NEST ECTOPARASITES				
Y=Total nest ectoparasites				
(1) Total nest ectoparasites ~ location + year	3402.93	0	0	NA
(1)Total nest ectoparasites~ colony + year	3397.12	0	0	NA
Y=Total nest fleas				
(1) Total fleas ~ location + year	3044.07	0	0	NA
(1) Total fleas ~ colony + year	2998.44	0	0	NA
Adult fleas				
(1) Adult fleas ~ location + year	1596.91	0	0	NA
(1)Adult fleas ~ colony + year	1556.82	0	0	NA
Y=Flea larvae				
(1) Flea larvae ~ location + year	2860.44	0	0	NA
(1) Flea larvae ~ colony + year	2802.76	0	0	NA
Y=Nest ticks				
(1) Nest ticks ~ location + year	2168.96			
(2) Nest ticks ~ location	2168.92	0.04	1	>0.05
(1) Nest ticks ~ colony + year	2146.11	0	0	NA

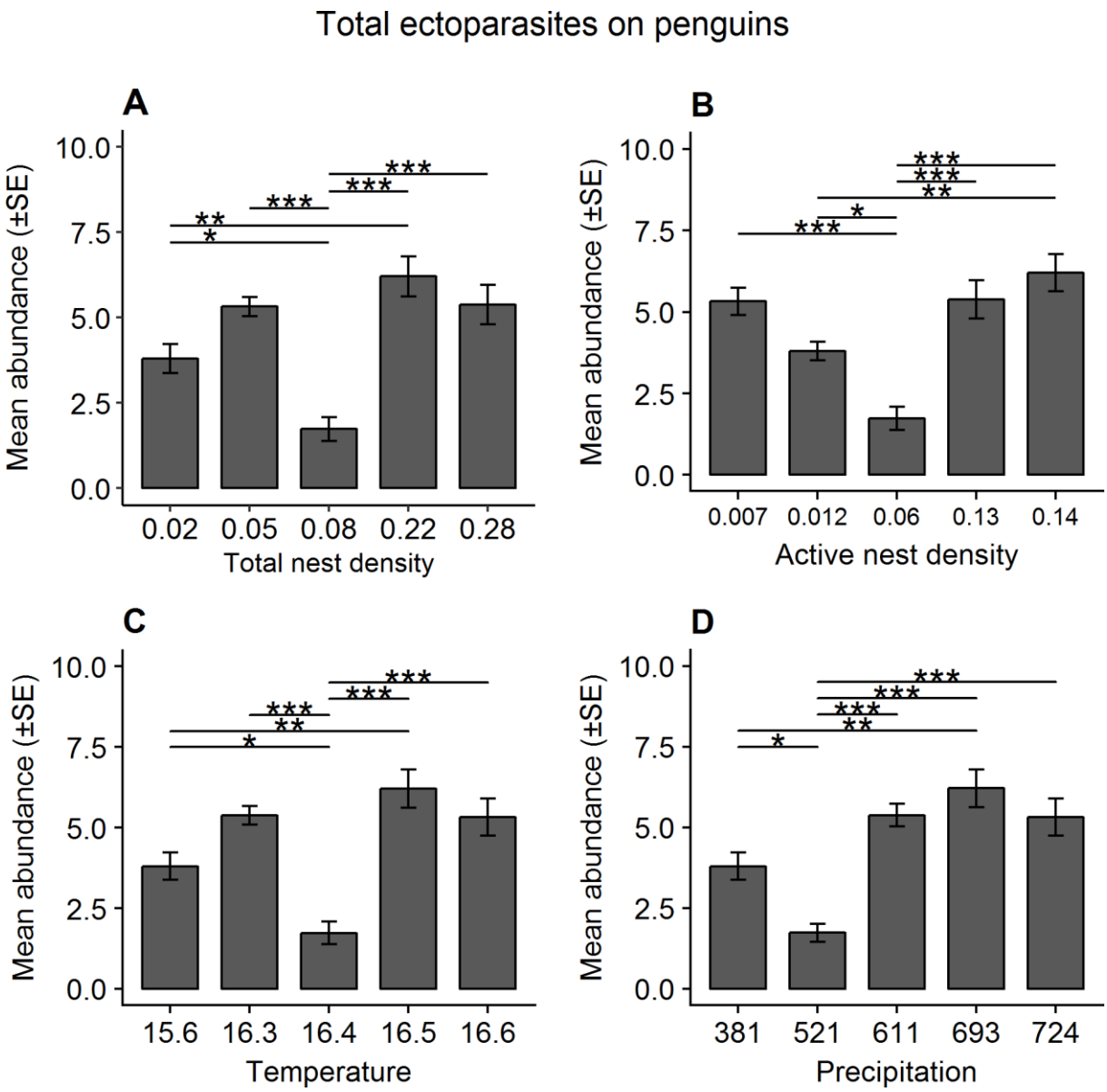

Figure S2.1. Cross-colony differences between mean abundance of total ectoparasites on penguins and (A) total nest density (average/m^2): Dassen Island 0.02, Robben Island 0.05, Dyer Island 0.08, Simon's Town 0.22, Stony Point 0.28; (B) active nest density (average/m^2): Robben Island 0.007, Dassen Island 0.012, Dyer Island 0.06, Stony Point 0.13, Simon's Town 0.14; (C) annual mean temperature (°C): Dassen Island 15.6, Stony Point 16.3, Dyer Island 16.4, Simon's Town 16.5, Robben Island 16.6; (D) annual precipitation (mm): Dassen Island 381, Dyer Island 521, Stony Point 611, Simon's Town 693, Robben Island 724.

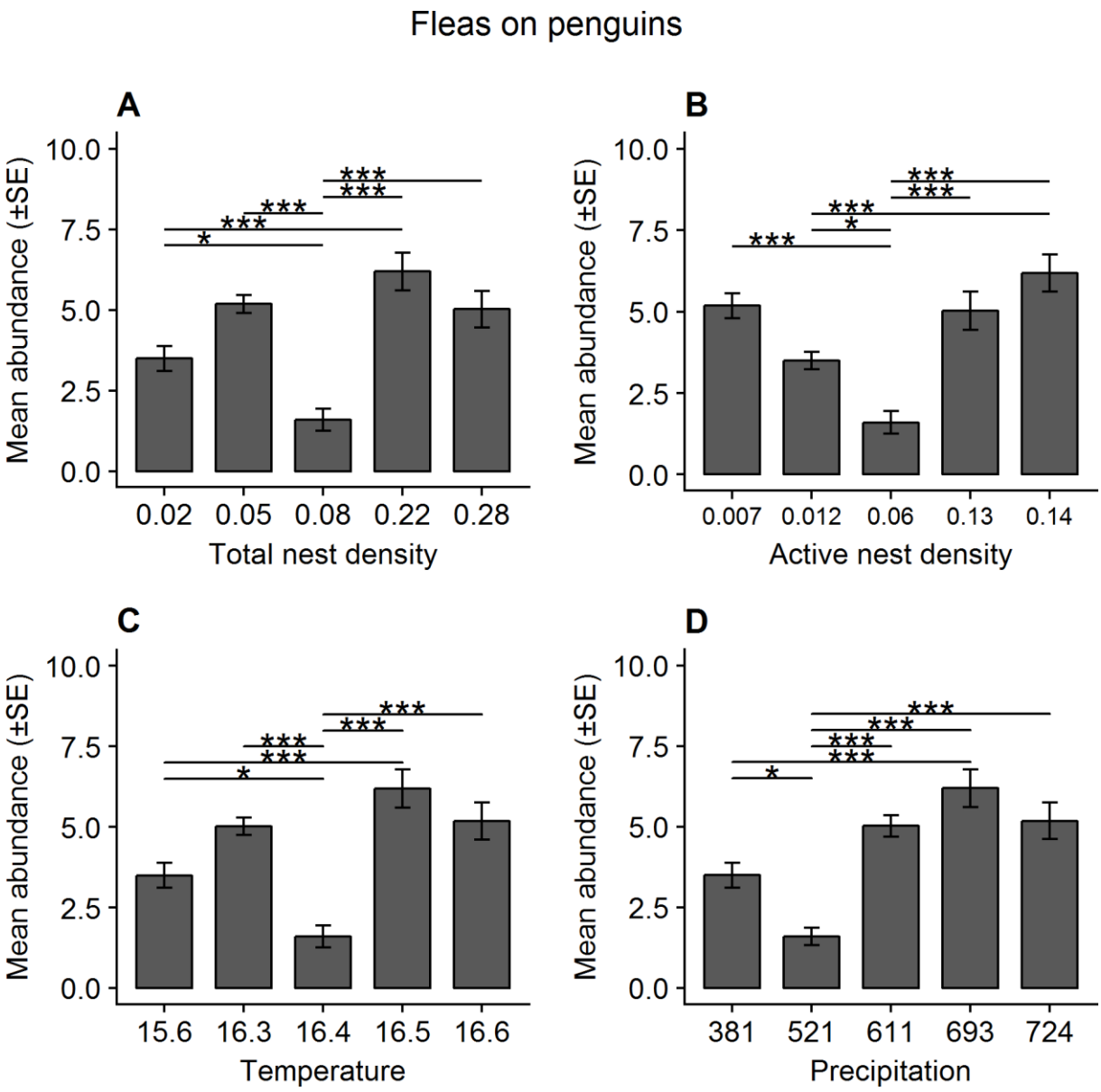

Figure S2.2. Cross-colony differences between mean abundance of fleas on penguins and (A) total nest density (average/m^2): Dassen Island 0.02, Robben Island 0.05, Dyer Island 0.08, Simon's Town 0.22, Stony Point 0.28; (B) active nest density (average/m^2): Robben Island 0.007, Dassen Island 0.012, Dyer Island 0.06, Stony Point 0.13, Simon's Town 0.14; (C) annual mean temperature (°C): Dassen Island 15.6, Stony Point 16.3, Dyer Island 16.4, Simon's Town 16.5, Robben Island 16.6; (D) annual precipitation (mm): Dassen Island 381, Dyer Island 521, Stony Point 611, Simon's Town 693, Robben Island 724.

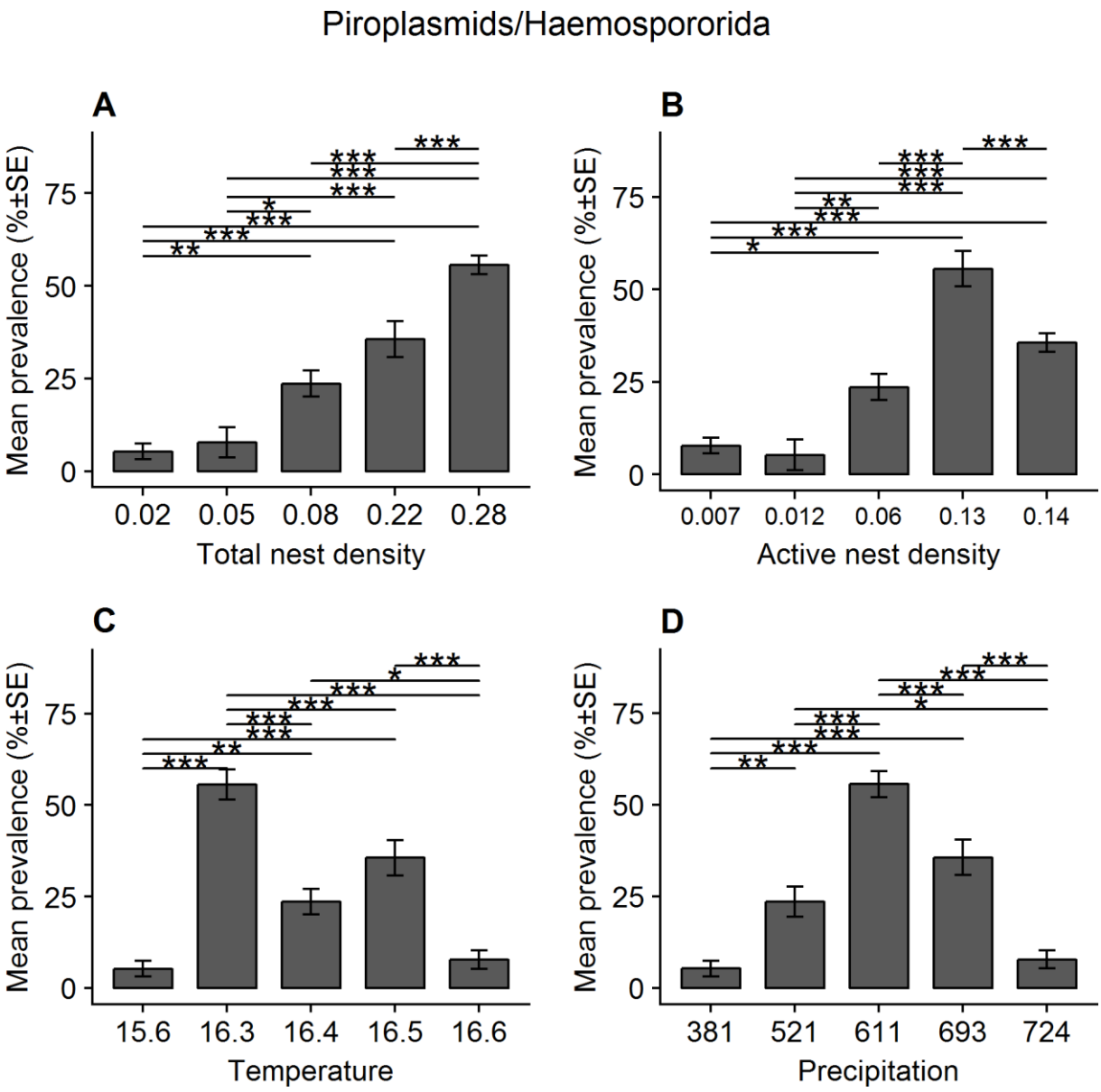

Figure S2.3. Cross-colony differences between mean prevalence of Piroplasmids/Haemosporida and (A) total nest density (average/m^2): Dassen Island 0.02, Robben Island 0.05, Dyer Island 0.08, Simon's Town 0.22, Stony Point 0.28; (B) active nest density (average/m^2): Robben Island 0.007, Dassen Island 0.012, Dyer Island 0.06, Stony Point 0.13, Simon's Town 0.14; (C) annual mean temperature (°C): Dassen Island 15.6, Stony Point 16.3, Dyer Island 16.4, Simon's Town 16.5, Robben Island 16.6; (D) annual precipitation (mm): Dassen Island 381, Dyer Island 521, Stony Point 611, Simon's Town 693, Robben Island 724.

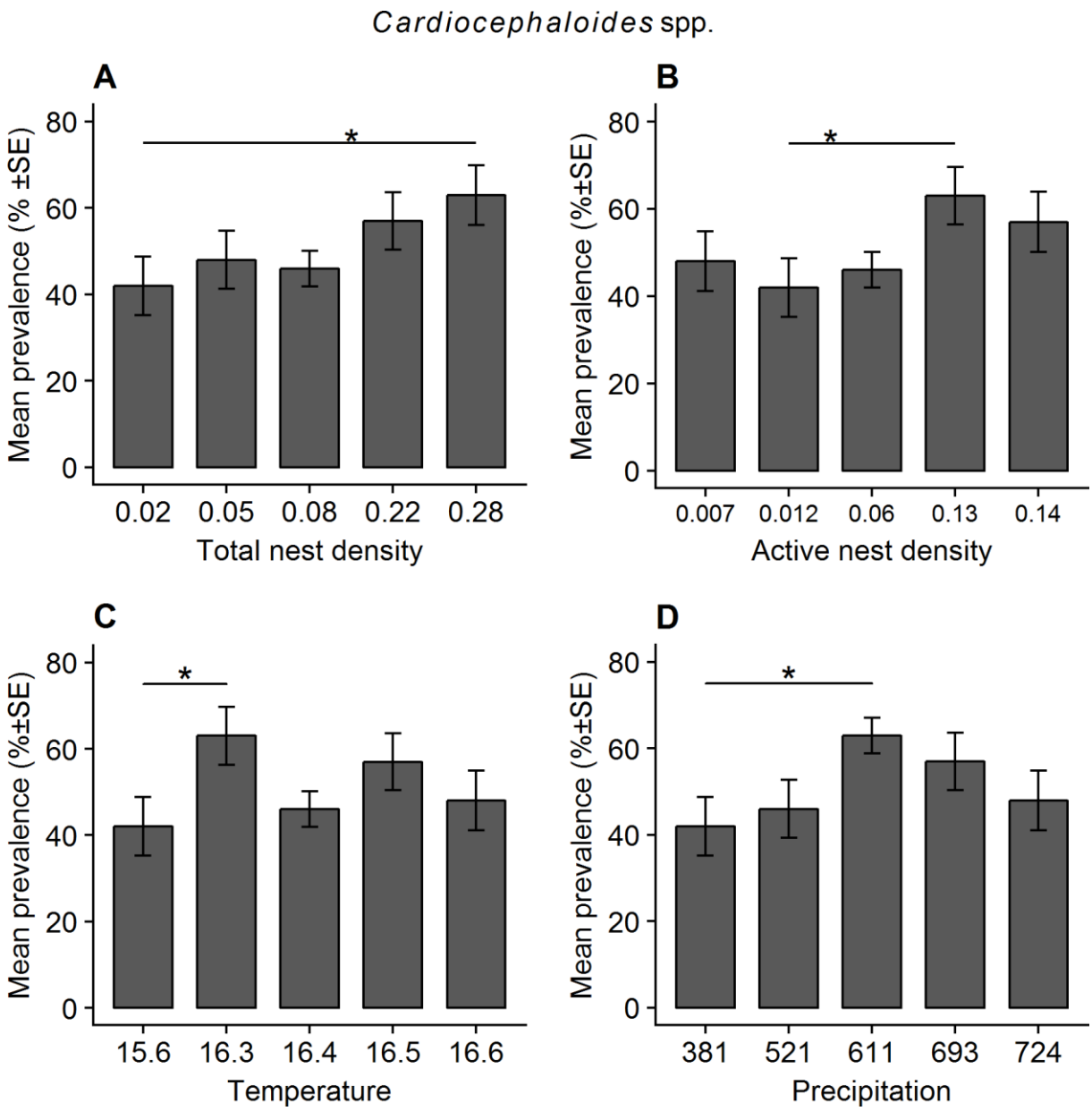

Figure S2.4. Cross-colony differences between mean prevalence of *Cardiocephaloides* spp. and (A) total nest density (average/m^2): Dassen Island 0.02, Robben Island 0.05, Dyer Island 0.08, Simon's Town 0.22, Stony Point 0.28; (B) active nest density (average/m^2): Robben Island 0.007, Dassen Island 0.012, Dyer Island 0.06, Stony Point 0.13, Simon's Town 0.14; (C) annual mean temperature (°C): Dassen Island 15.6, Stony Point 16.3, Dyer Island 16.4, Simon's Town 16.5, Robben Island 16.6; (D) annual precipitation (mm): Dassen Island 381, Dyer Island 521, Stony Point 611, Simon's Town 693, Robben Island 724.

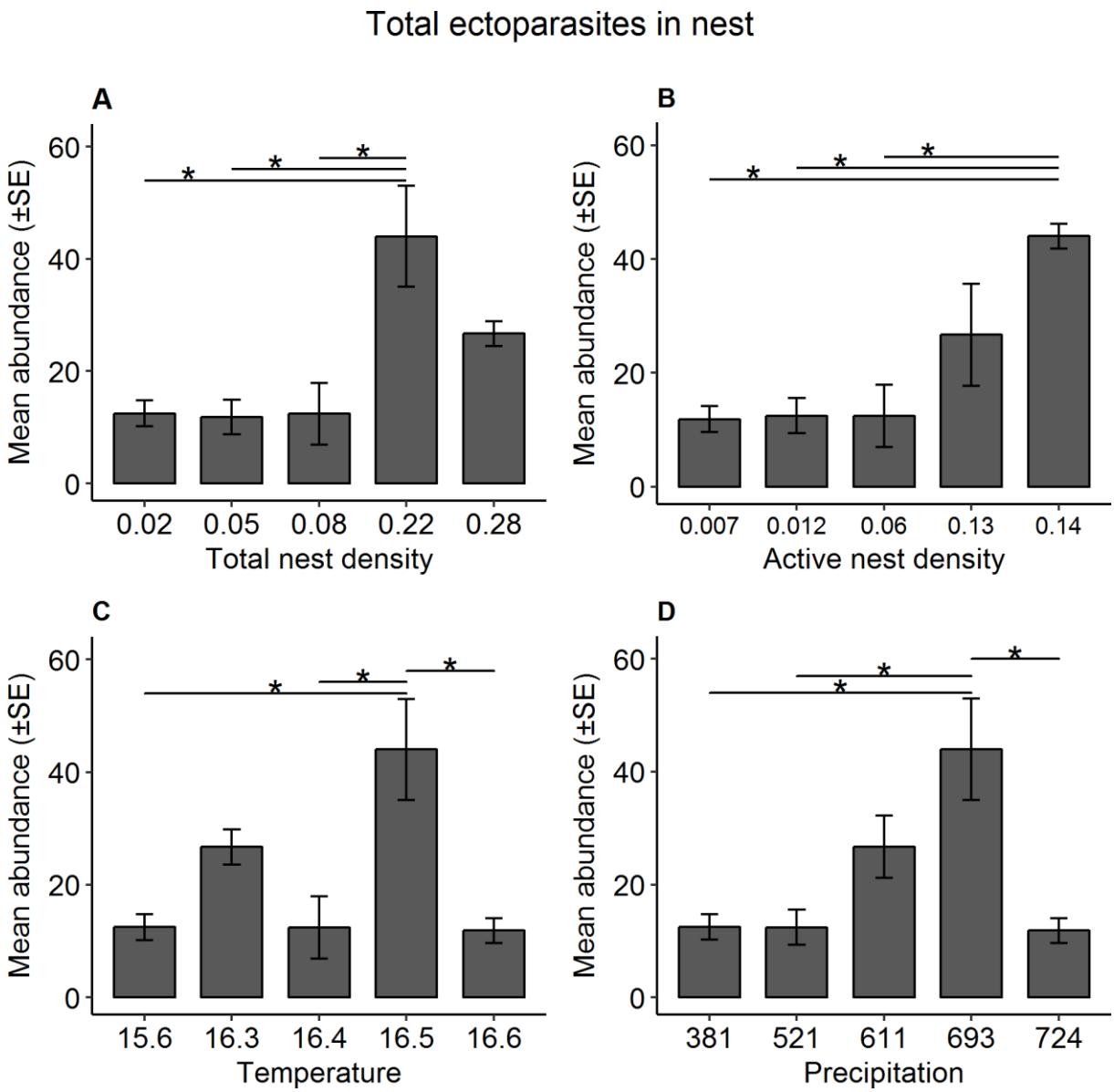

Figure S2.5. Cross-colony differences between mean abundance of total nest ectoparasites and (A) total nest density (average/m^2): Dassen Island 0.02, Robben Island 0.05, Dyer Island 0.08, Simon's Town 0.22, Stony Point 0.28; (B) active nest density (average/m^2): Robben Island 0.007, Dassen Island 0.012, Dyer Island 0.06, Stony Point 0.13, Simon's Town 0.14; (C) annual mean temperature (°C): Dassen Island 15.6, Stony Point 16.3, Dyer Island 16.4, Simon's Town 16.5, Robben Island 16.6; (D) annual precipitation (mm): Dassen Island 381, Dyer Island 521, Stony Point 611, Simon's Town 693, Robben Island 724.

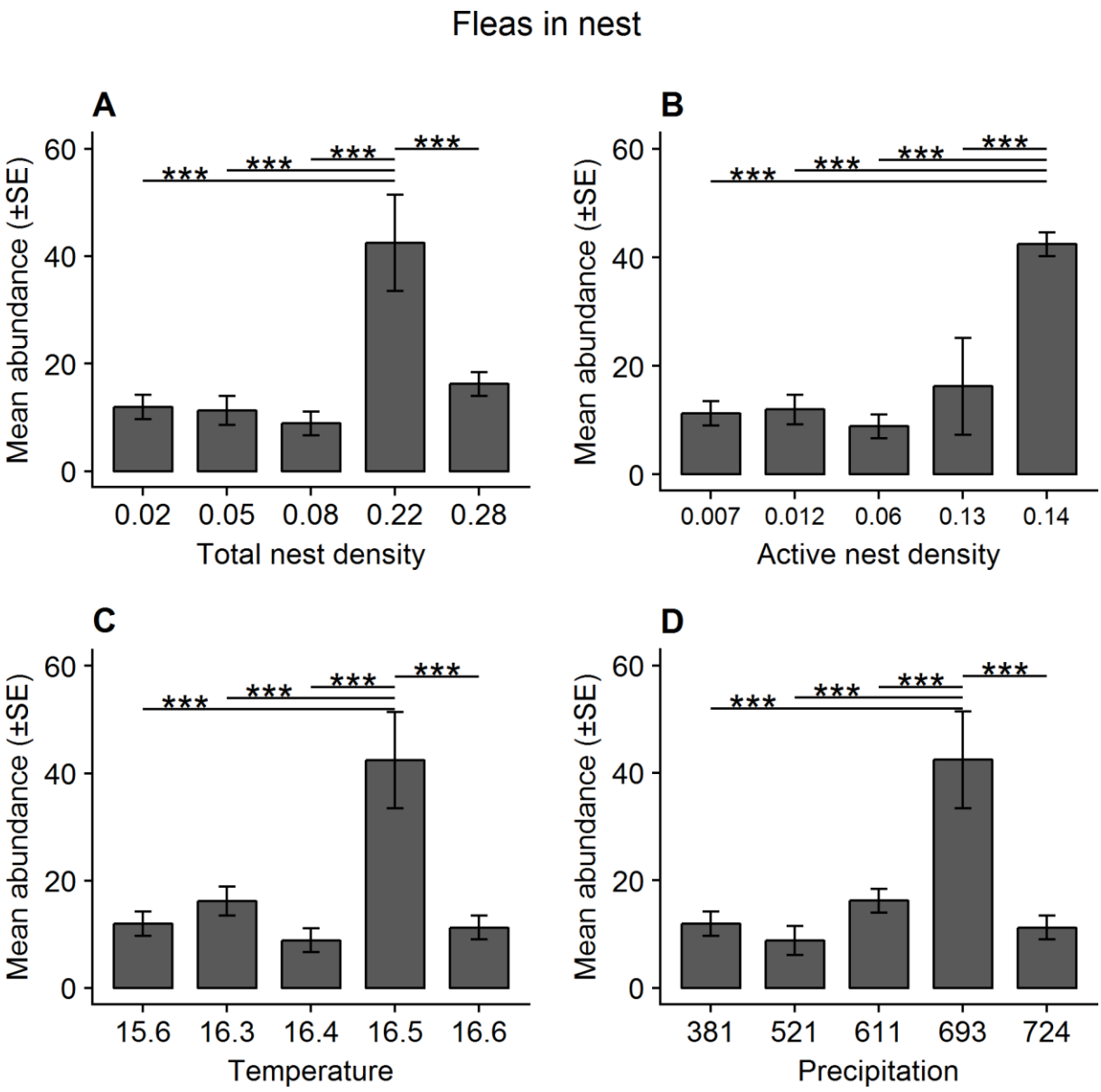

Figure S2.6. Cross-colony differences between mean abundance of nest fleas and (A) total nest density (average/m^2): Dassen Island 0.02, Robben Island 0.05, Dyer Island 0.08, Simon's Town 0.22, Stony Point 0.28; (B) active nest density (average/m^2): Robben Island 0.007, Dassen Island 0.012, Dyer Island 0.06, Stony Point 0.13, Simon's Town 0.14; (C) annual mean temperature (°C): Dassen Island 15.6, Stony Point 16.3, Dyer Island 16.4, Simon's Town 16.5, Robben Island 16.6; (D) annual precipitation (mm): Dassen Island 381, Dyer Island 521, Stony Point 611, Simon's Town 693, Robben Island 724.

Chapter 3

Health evaluation of wild African penguins (*Spheniscus demersus*) and the potential impact of parasites

(Prepared for *Journal of Avian Biology*)

Abstract

Baseline data on clinical parameters of the endangered African penguin have mostly been conducted on adult birds that were admitted to rehabilitation centres. As such, it is uncertain if these clinical parameters are a true reflection of African penguins in their natural environment. We assessed clinical parameters (body mass, body condition of chicks, haematocrit and total plasma protein) and established the effect of on-host ectoparasites, haemoparasites, and helminth parasites on 793 African penguins (210 adults and 583 chicks) in five African penguin colonies along the south-western coast of South Africa during autumn/winter and spring of 2016 and autumn/winter of 2017. Clinical parameters varied between colonies, but generally fell within the reference ranges presented by previous studies. Penguin body condition (chicks) was significantly lower and haematocrit (adults and chicks) significantly higher at Dassen Island, while penguins at Simon's Town and Stony Point recorded significantly lower haematocrit values. Chick body condition was significantly lower in spring compared to autumn/winter. Ecto- and haemoparasite species richness was negatively related to haematocrit values, but helminth species richness was positively related to body mass and haematocrit. In the Stony Point colony, tick abundance and ecto- and haemoparasite species richness were associated with lower haematocrit values. The full complement of factors that shape interspecific variation in clinical parameters between colonies are as yet not realised, however it is evident that the incidence of parasite infestations must be considered.

Keywords: Clinical parameters, penguin health, penguin parasites, African penguin.

Introduction

Wildlife health assessments are increasingly gaining attention among conservationists as a tool to assess the health status of wild populations (Deem et al. 2001). Although the inclusion of health assessments as part of monitoring programmes is advisable for all wildlife, it is particularly of value in the management and conservation of species that are at risk of extinction (Karesh and Cook 1995). The success of this tool depends on the availability of baseline haematology and morphometric data for healthy wild populations, which is used as reference data to compare and assess the status of the relevant test populations (Karesh et al. 1999; Parsons et al. 2015; Kloskowski et al. 2016). Monitoring the health status of wildlife in natural environments can thus act as an early-warning system for potential stress factors in the environment and facilitate the detection of pathogenic agents (Averbeck 1992). In particular, extreme stressful events, such as chronic exposure to environmental pollution, dehydration, food deficiency, disease and human disturbance, are some of the factors that can change the clinical parameters in animals (Vleck et al. 2000, Fair et al. 2007, Norte et al. 2009).

Specifically, studies on wild bird populations recorded that parasite infections are a common cause of variation in the health status of birds, which can potentially have a detrimental effect on their hosts (Quillfeldt et al. 2004, Campbell and Ellis 2007). For example, high infestations of the hard tick *Ixodes uriae* were negatively related with haematocrit levels in black-legged kittiwake (*Rissa tridactyla*) (Wanless et al. 1997). In addition, haemosporidian haemoparasites, such as *Plasmodium* spp. and *Leucocytozoon* spp., produced haemolytic anemia in bird species worldwide (Atkinson and van Riper III 1991, Atkinson 2008), while *Haemoproteus* spp. was associated with lower mean body mass and fat reserves in Neotropical migrant passerines (Garvin et al. 2006). Furthermore, moderate to heavy burdens of helminth parasites, including gastric nematodes and intestinal trematodes, were associated with poor body condition and depleted fat reserves in little penguins (*Eudyptula minor*) in Australia (Obendorf and McColl 1980).

The African penguin (*Spheniscus demersus*) is a seabird species endemic to southern Africa (Crawford et al. 2006a). The species experienced a dramatic population decline (>50% in three generations) in recent years to the extent that its conservation status is listed

as endangered on the IUCN red list (BirdLife International 2016), with the population continuing to decline. Various anthropogenic linked factors contributed to the decline, but it is surmised that fishing pressure (Coetzee et al. 2008) together with changes in the marine environment (Roy et al. 2007) in the southern Benguela Upwelling Ecosystem have resulted in an eastward shift of important food resources such as sardine and anchovy (Fairweather et al. 2006, Crawford et al. 2007). This displaced the stocks outside the foraging range of breeding penguins (Crawford et al. 2006b, 2008a, b), causing these seabird population declines (Crawford et al. 2011). African penguins currently breed in 28 colonies distributed between the coast of central Namibia to the east coast of South Africa (Crawford et al. 2011, BirdLife International 2016). As part of the managing authorities' ecological monitoring programmes, penguin colonies are actively monitored in an attempt to identify and mitigate any threats to the survival of the species. To further aid in the conservation of the species a species management plan, the African Penguin Biodiversity Management Plan (Department of Environmental Affairs 2013), was drafted to highlight the necessary actions that would contribute to the long term survival of the species in the wild. A health assessment, which would establish baseline haematology and biochemistry parameters for healthy birds as well as determine the current health status of the colonies was recommended. These health guidelines were developed based on studies conducted across several major colonies, regions and years (Lubbe et al. 2014; Parsons et al. 2015, 2016). However, although baseline health parameters do exist for penguin chicks there is a bias towards adult birds (Parsons et al. 2015, 2016). African penguins are host to a large diversity of parasites that include ectoparasites (fleas, soft ticks and lice), haemoparasites (parasitic bacteria, protozoa and virus) and helminth parasites (nematodes, trematodes and cestodes) (Parsons and Vanstreels 2016). Although parasites form part of the natural ecosystem, it is possible that elevated infestations or multi-species infections can adversely affect the health of African penguins (Parsons et al. 2016). The present study aims to: 1) Assess basic clinical parameters (body mass, body condition (for chicks), haematocrit and total plasma protein) of wild adult and chick African penguins across five colonies along the south-western coast of South Africa, and 2) Establish the effect of on-host parasite infestations (ecto-, haemo- and helminth parasites) on these clinical parameters.

Methods

Study design

This study was conducted at five colonies along the south-western coast of South Africa in 2016 and 2017 (Fig. 3.1, Table 3.1). During the peak breeding season (autumn/winter; May to July) adult penguins and chicks (>20 day-old and in nests) were randomly selected throughout the colonies. A total of 210 African penguins (42 adults and 168 chicks) were sampled at Stony Point and 120 African penguins (40 adults and 80 chicks) were sampled at each one of the other four colonies. In addition, a second sampling season was conducted at Stony Point in spring (October-November) in 2016 when 103 penguins (8 adults and 95 chicks) were sampled (Table 3.1). The sample size for adult penguins in spring 2016 was low because spring coincides with the moulting season of adult penguins (September to January for penguins in the Western Cape (Crawford et al. 2006c), and therefore a large number of adult penguins were not present in the nests during this sampling period.

Morphometric and haematological sample collection and processing

Birds that were selected were sitting in the nests. Only visually healthy and unstressed animals were selected for the study. Each bird was restrained for 8 minutes and upon completion released at the capture site. Body mass (kg) was recorded for each penguin with a handheld electronic scale (25kg/50lb Sensation). For chicks, head length (mm) was recorded from the tip of the culmen to the back of the skull (Lubbe et al. 2014) with an electronic calliper (Grip 150mm Digital Vernier). Head length and body mass were used to calculate a body condition index (Lubbe et al. 2014) based on maximum and minimum growth relative to structural size. Since head length increases linearly with body mass in growing birds, the body condition index is suitable for chicks only. Blood was collected from the dorsal aspect of the foot, using a 23-gauge needle, and placed in 80 ul heparinized microhematocrit capillary tubes. Capillary tubes were centrifuged (on site and on the same day) using a portable centrifuge (Hawksley & sons Ltd.) at 14000 rpm for 5 minutes (Travis et al. 2006). Due to logistical problems we could not conduct the latter part for penguins at Dassen Island in 2016. Haematocrit (packed cell volume) was measured using a microhematocrit reader (Hawksley & sons Ltd.) and total plasma protein (TPP) was

determined using a handheld refractometer. We initially used a Bellingham and Stanley Ltd. refractometer in 2016 (on loan) and in 2017 we used a Schmidt + Haensch refractometer. In all cases the refractometer was calibrated before use.

Parasite collection and identification

The methods used to collect and identify the ecto-, haemo- and helminth parasites are provided in detail in Espinaze et al. (in review) (Chapter 2). In short, ectoparasites (fleas, lice and ticks) were collected by systematically brushing the plumage of each penguin. Haemoparasites (Piroplasmorida/Haemosporida and Spirochaetales) were recorded using thin blood smears (fixed with methanol and stained with Eosin-Methylene Blue stain (RapiDiff kit). Helminth parasites were recorded, for chicks only, using fresh faecal material (1 g). Nematode eggs were detected with the modified Wisconsin sugar flotation method (Nolan 2006) and the sedimentation technique (Hansen and Perry 1994) was used to detect trematode eggs. Ectoparasites were identified to species level, using taxonomic reference keys (Bedford 1934, Jordan 1942; Von Keler 1952, Arthur 1963, Kohls et al. 1965, Segerman 1995, Banks and Palma 2003) and life stage and sex were recorded. Haemoparasites were identified to order level based on morphological characters (Earlé et al. 1993, Campbell and Ellis, 2007, Peirce and Parsons 2012, Vanstreels et al. 2016) and helminth parasites were identified to genus level (Horne et al. 2011, Carrera-Játiva et al. 2014, Viljoen 2015).

Data analysis

The clinical parameters that were examined were body condition, haematocrit, TPP and body mass. Because clinical parameters are influenced by penguin age (Cherel et al. 1993, Merino and Barbosa 1997, Travis et al. 2006), we separately presented values obtained from adult penguins and chicks. Mean and standard deviation values obtained from clinical parameters were compared across colonies and contrasted with those found for healthy African penguins (reference values for wild adult penguins in Parsons et al. 2015; reference values for chicks sampled within three days of admission at the Southern African Foundation for the Conservation of Coastal Birds (SANCCOB) in Parsons et al. 2016).

Only TPP were compared differently because there are no published values of TPP recorded from wild African penguins. We therefore used values of total serum proteins, but considered that protein in plasma is about 0.1 g/dl higher than protein in serum (Lumeij 1987). All data was tested for normality using the Shapiro-Wilk test. Statistical differences between data distribution at each colony were determined with a Kruskal-Wallis non-parametric test and compared with a Dunn`s Multiple Comparison Test. Parasite abundance (i.e. the total number of individuals per parasite species in a penguin) and richness (i.e. number of parasite species parasitizing a penguin) were compared across colonies. Regression models were used to assess the potential effect of parasite abundance, parasite richness, and colony, correcting for penguin age (adult and chicks) and year (2016 and 2017), on clinical parameters of penguins during autumn\winter 2016 and 2017. Since helminth parasites were collected only from chicks, the regression models that include body condition and helminth parasites do not consider penguin age as a variable. When colony was used as a categorical variable in the regression models, we rotated each colony as the reference but chose only to report results with the Simon's Town colony as the reference for simplicity. We ran a separate analysis on the data from Stony Point to assess the effect of season (autumn/winter vs. spring) on the body condition of chicks. We ran a generalised linear model (GLM) using the glm() function with a Gaussian error distribution for body condition and TPP data, and a GLM with a Poisson (or a negative binomial when necessary) error distribution for haematocrit and body mass. Since we aimed at assessing the effect of different factors on clinical parameters of penguins, we presented the full models with all independent variables in the main text. However, we also performed backward model selection based on Akaike information criterion (AIC), using the function step() in R and compared the selected models with the corresponding full models using a Chi-square test. All statistical tests were carried out with using in R3.4.3 (R Core Team 2017).

Results

Clinical parameters of African penguins across colonies

A mean body mass of 2.71 kg (±0.36) was recorded for adult penguins and 2.05 kg (±0.68) for chicks across colonies during the autumn/winter season of 2016 and 2017. The mean body mass for adult penguins and chicks differed significantly between the individual colonies (Kruskal-Wallis=13.393, $p<0.01$ and Kruskal-Wallis=21.842, $p<0.001$, respectively) (Fig. 3.2a and 3.2b). At all colonies with the exception of Dyer Island and Stony Point, the mean body mass values for adults per colony were lower but still within the references range recorded for healthy adult African penguins in the Western Cape (2.80 kg ±0.37; Parsons et al. 2015). The mean body mass values for chicks were lower compared to the references range for healthy African penguin chicks (2.32 kg ±0.3; Parsons et al. 2016). In particular, chicks at Dyer Island and Simon's Town (Fig. 3.2b) recorded a lower mean body mass compared to penguins at the other colonies.

A mean body condition value of 0.43 (±0.41) was recorded for chicks across colonies during the autumn/winter season of 2016 and 2017 (Fig. 3.2c). The mean body condition for chicks differed significantly (Kruskal-Wallis=22.664, $p<0.001$) between the individual colonies, but the values still fell within the references range described for healthy chicks (0-1 body condition index; Lubbe et al. 2014). However, the body condition for chicks at Dassen Island (0.28±0.35) was lower compared to the other colonies (*t*-statistic $p<0.05$). When comparing between different sampling seasons, the mean body condition of chicks was significantly lower (*t*-statistic $p<0.001$) in spring (-0.21 ±0.54) compared to autumn/winter (0.48 ±0.33) at Stony Point in 2016. Further, the values recorded for chicks in spring were generally below the reference range for healthy chicks (Fig. 3.2d).

A mean haematocrit value of 47.71% (±5.34) was recorded for adult penguins and 31.82% (±5.62) for chicks across colonies during the autumn/winter season of 2016 and 2017. In most cases the values for adult penguins and chicks fell within the references range for healthy adult African penguins (46% ±6.70; Parsons et al. 2015) and healthy chicks in the Western Cape (31% ±6.4, Parsons et al. 2016), respectively (Fig. 3.3a and 3.3b). The mean haematocrit values for adult penguins and chicks differed significantly between the individual colonies (Kruskal-Wallis=28.888, $p<0.001$ and Kruskal-Wallis=66.935, $p<0.001$, respectively). In both age groups the mean haematocrit value was higher at Dassen Island compared to other colonies.

A mean TPP value of 8.4g/dl (±1.2) was recorded for adult penguins and 7.1g/dl (±1.1) for chicks across colonies during the autumn/winter season of 2016 and 2017. The mean TPP values at individual colonies was higher than the reference values of total serum protein described for healthy adult African penguins (5.9g/dl ±0.96; Parsons et al. 2015) and healthy chicks (4.31g/dl ±0.54; Parsons et al. 2016) (Fig. 3.3c and 3.3d). For both age groups, the mean TPP differed significantly between the individual colonies (adults: Kruskal-Wallis=10.875, $p<0.05$; and chicks: Kruskal-Wallis=15.604, $p<0.01$). In addition, Dassen Island recorded the lowest mean TPP values for both age groups compared to the other colonies.

Parasite species abundance and richness

Three parasitic groups were recorded from the bodies of 690 African penguins at the five colonies along the south-western coast of South Africa: 1) Ectoparasites included two fleas (*Parapsyllus humboldti* and *Echidnophaga gallinacea*), a soft tick (*Ornithodoros capensis*), and a louse (*Austrogoniodes demersus*); 2) Haemoparasites from the orders Piroplasmorida/Haemospororida and Spirochaetales; and 3) Helminth parasites from four genera *Cardiocephaloides* spp., *Renicola* spp., *Contracaecum* spp. and *Cyathostoma* spp. in the faecal material of penguin chicks. Simon's Town recorded the highest on-host abundance of fleas and Stony Point the highest on-host tick abundance, while Simon`s Town was the only colony where lice were not found (Table 3.2). Most of the parasite species were found across the colonies during the autumn/winter season, except for *E. gallinacea* which was only found on Dassen Island, and *Renicola* spp. which was found during spring in Stony Point.

Factors that affect clinical parameters of African penguins

The potential effect of on-host parasite abundance and species richness on clinical parameters of African penguins during the autumn/winter season across the selected colonies is presented in Table 3.3. The majority of the (full) models included in the analysis did not show significant differences compared to the best models estimated with the AIC (Supplementary Table S3.1). Overall, there was no significant relationship between on-host

fleas, ticks or lice abundance and the mean body mass, haematocrit and TTP of penguins (age groups combined), and the body condition of penguin chicks. However, parasite species richness had a variable effect on the clinical parameters. In particular, ectoparasite species richness had a significant negative relationship with penguin body mass (*z*-statistic $p<0.05$), while ecto- and haemoparasite species richness had a significant negative relationship with the haematocrit of penguins (*z*-statistic $p<0.01$ and *z*-statistic $p<0.001$, respectively). In contrast, helminth species richness had a significant positive relationship with the body mass (*z*-statistic $p<0.01$) and haematocrit (*z*-statistic $p<0.05$) of penguin chicks (Table 3.3).

Results from the generalised linear models largely support between colony differences in clinical parameters. Colony differences were significant for mean body mass with penguins at Stony Point recording significantly higher mean body mass compared to Simon`s Town (*z*-statistic $p<0.01$), Dassen Island (*z*-statistic $p<0.05$) and Dyer Island (*z*-statistic $p<0.001$). Chick body condition was poorer (lower) at Dassen Island compared to all of the other colonies (*t*-statistic $p<0.01$). Penguins at Simon`s Town (*z*-statistic $p<0.001$) and Stony Point (*z*-statistic $p<0.01$) had a lower haematocrit compared to Dassen-, Dyer- and Robben Island, while penguins at Dassen Island (*z*-statistic $p<0.001$) had the highest haematocrit compared to all the other colonies.

The potential effect of parasite abundance and species richness on clinical parameters of African penguins at Stony Point during the autumn/winter and spring season is presented in Table 3.4. None of the (full) models included in the analysis showed significant differences compared to the best models estimated with the AIC (Supplementary Table S3.2). On-host tick abundance had a significant negative relationship with the haematocrit of penguins (*z*-statistic $p<0.05$). This was the only potential effect of parasite abundance on the clinical parameters recorded at Stony Point. Likewise, the species richness of ecto- and haemoparasites was negatively related to haematocrit values (*z*-statistic $p<0.5$ and *z*-statistic $p<0.01$, respectively). All clinical parameters for penguins (combined ages) and chicks at Stony Point were significantly negatively related with season (*z*-and *t*-statistic $p<0.001$ in all cases).

Table 3.1. Locality, date of sampling, sample size and sampling season of the five African penguin colonies along the south-western coast of South Africa, during 2016 and 2017.

Locality	Coordinates	Sampling date		Sample size (adult:chicks)	Season
		2016	2017		
Island-based colonies					
Dassen Island	33.423647S, 18.086542E	12 May - 14 May	08 May - 12 May	120 birds (40:80)	autumn/winter
Dyer Island	34.684075S, 19.414769E	30 May - 01 June	29 July - 31 July	120 birds (40:80)	autumn/winter
Robben Island	33.807607S, 18.371231E	07 June - 26 June	29 May - 02 June	120 birds (40:80)	autumn/winter
Land-based colonies					
Simon's Town	34.197220S, 18.451285E	13 June - 20 June	26 June - 03 July	120 birds (40:80)	autumn/winter
Stony Point	34.374151S, 18.895248E	29 June - 13 July	19 June - 12 July	210 birds (42:168)	autumn/winter
		24 October - 07 November		103 birds (8:95)	spring

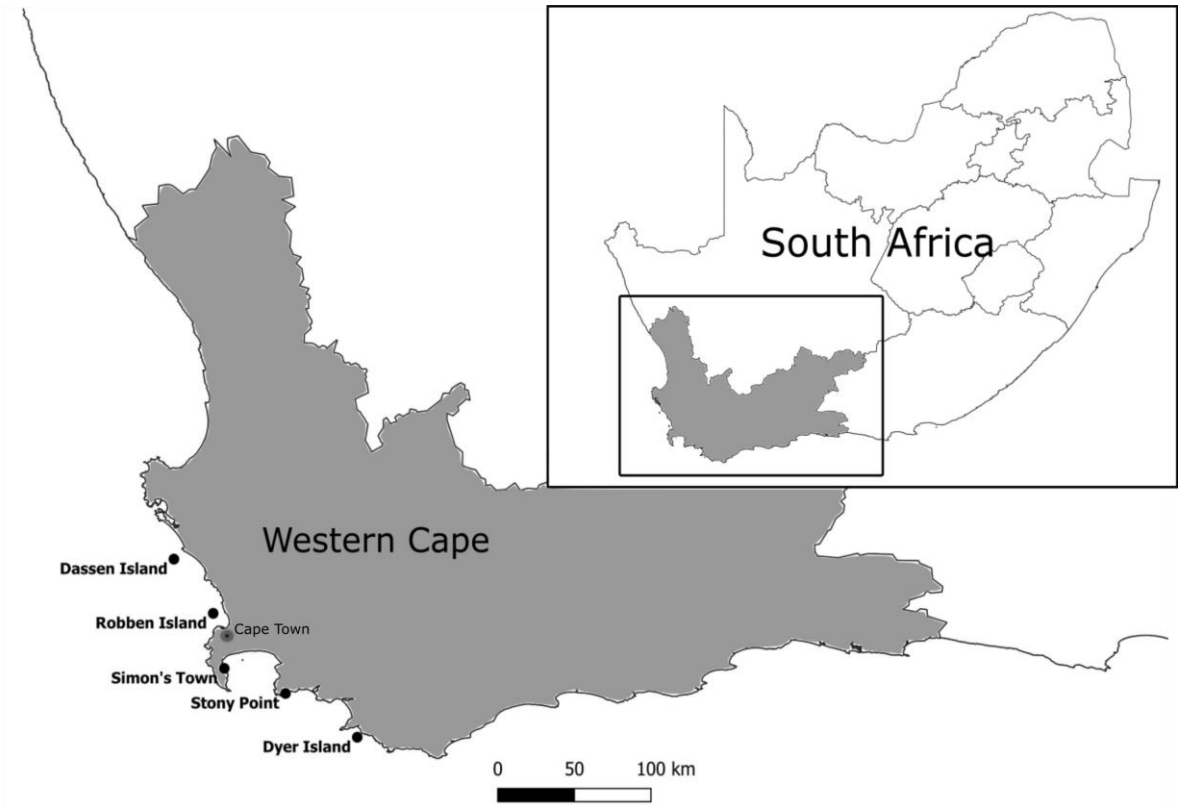

Figure 3.1. Map of the five selected African penguin colonies along the south-western coast of South Africa that were sampled during 2016 and 2017.

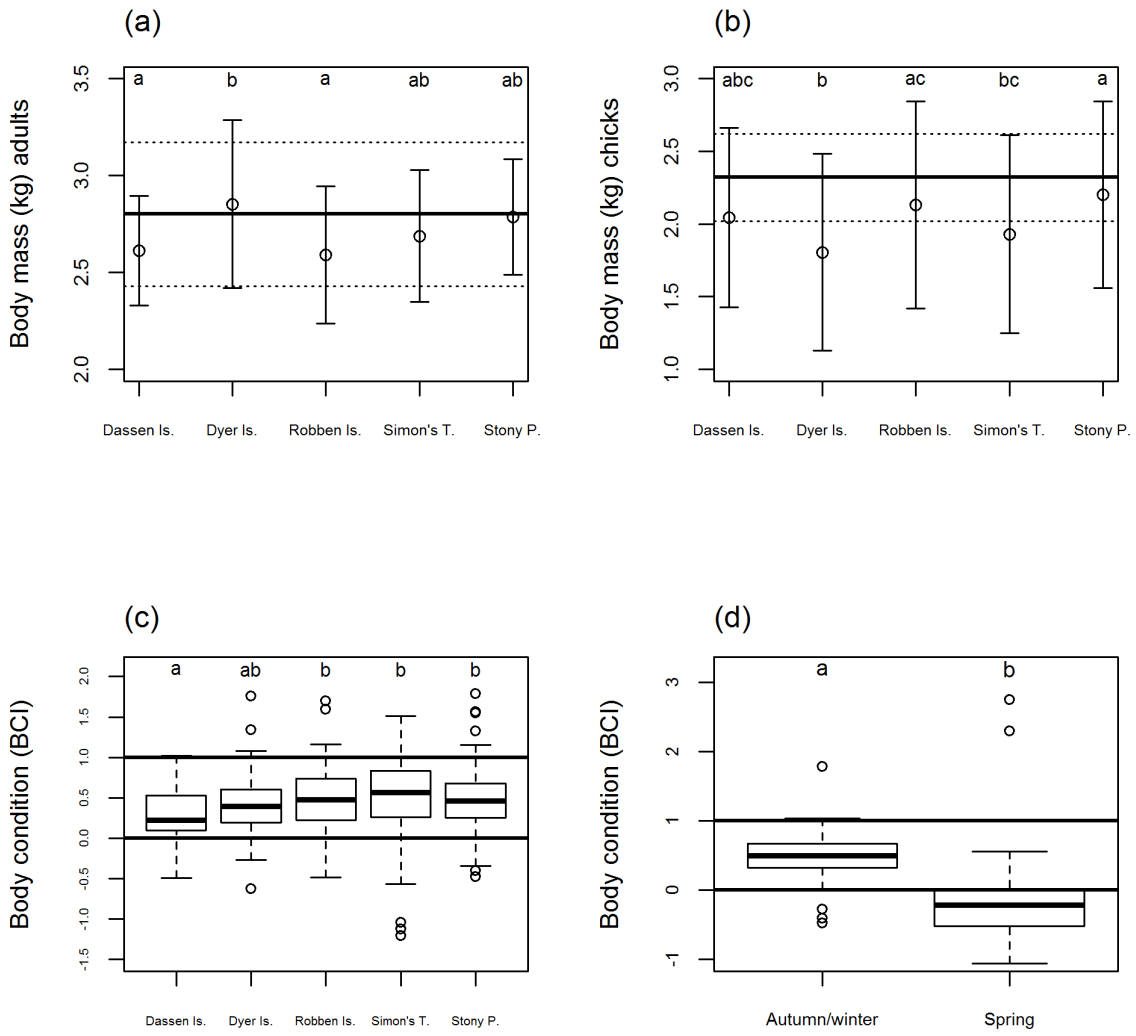

Figure 3.2. Comparison of clinical parameters of African penguins between colonies and seasons. Mean body mass (±SD) recorded from adults (a) and chicks (b) of African penguins across five colonies in the autumn/winter season 2016 and 2017. Solid horizontal lines represent the mean and dotted horizontal lines the standard deviation of reference values of body mass from healthy penguins (Parsons et al. 2015, 2016). Box plot of chick body condition across colonies in the autumn/winter season 2016 and 2017 (c) and at Stony Point in the autumn/winter and spring seasons 2016 (d). Solid horizontal lines represent the reference range of values for healthy chicks (Lubbe et al. 2014). Colonies not sharing letters were statistically different (Dunn`s Multiple Comparison Test).

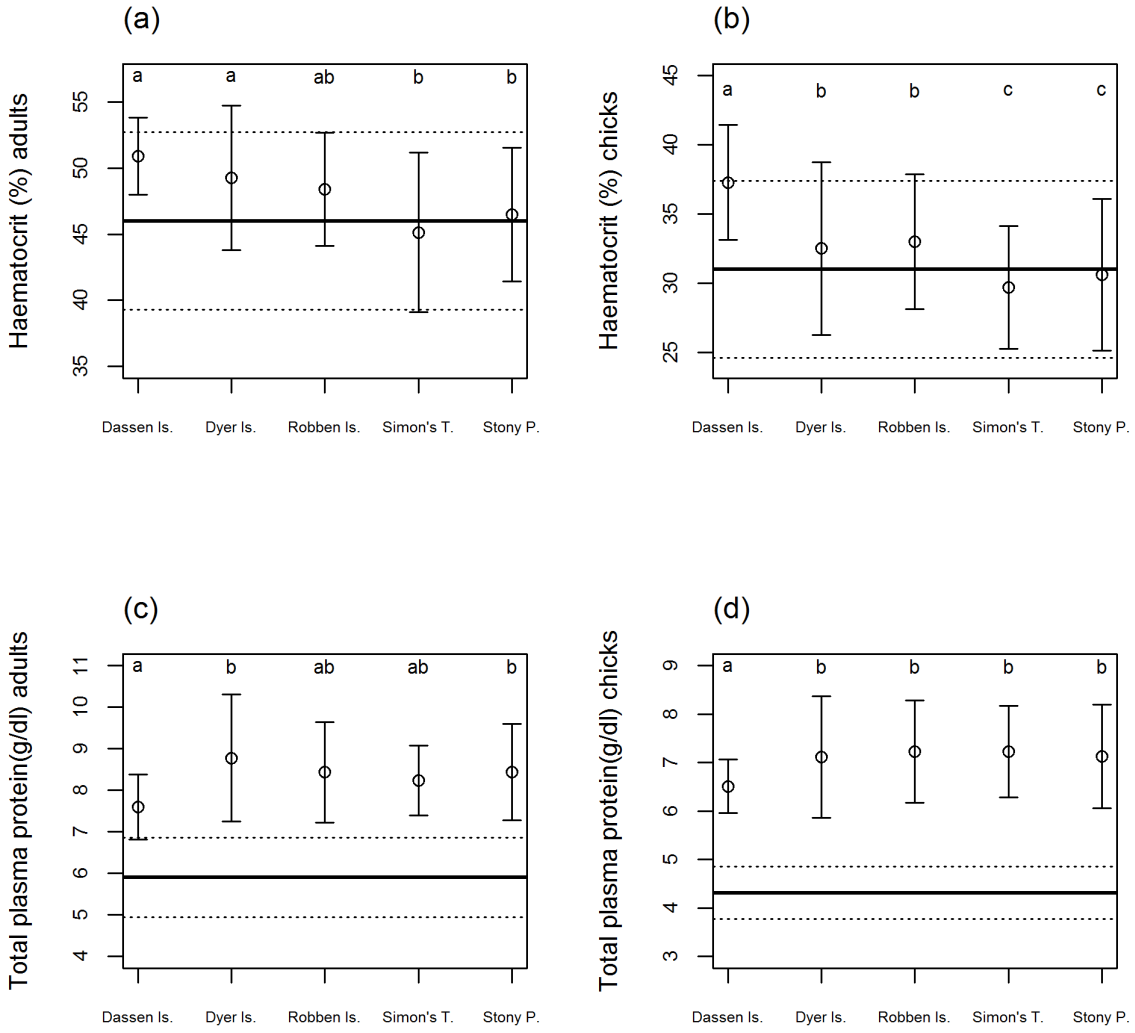

Figure 3.3. Comparison of clinical parameters of African penguins between colonies. Mean haematocrit (±SD) recorded from adult (a) and chick (b) African penguins, and mean total plasma protein (±SD) recorded from adults (c) and chicks (d) across five colonies in the autumn/winter season 2016 and 2017. Solid horizontal lines represent the mean and dotted horizontal lines the standard deviation of reference values of haematocrit and total serum protein from healthy penguins (Parsons et al. 2015, 2016). Colonies not sharing letters were statistically different (Dunn`s Multiple Comparison Test).

Table 3.2. Mean abundance (±SE) and richness (number of species) of parasites (on-host ecto-, haemo-, and helminth parasites) recorded from African penguins between five colonies in the autumn/winter season 2016 and 2017. Sample sizes: 690 penguins (adults and chicks) for ectoparasites, 641 penguins for haemoparasites and 367 chicks for helminth parasites.

Penguin colonies		Dassen Island	Dyer Island	Robben Island	Simon's Town	Stony Point
Mean abundance (±SE)	Fleas	3.50 (±0.39)	1.6 (±0.27)	5.18 (±0.57)	6.19 (±0.59)	5.02 (±0.34)
	Ticks	0.25 (±0.18)	0.12 (±0.06)	0.13 (±0.04)	0.02 (±0.01)	0.35 (±0.08)
	Lice	0.05 (±0.04)	0.02 (±0.01)	0.01 (±0.01)	0	0.01 (±0.01)
Species richness	Ectoparasites	4	3	3	2	3
	Haemoparasites	2	2	2	2	2
	Helminth parasites	2	3	2	2	3

Table 3.3. Relationship between on-host parasite abundance (fleas, ticks and lice), parasite richness (ecto-, haemo-, and helminth parasites species), colony (Simon`s Town, Stony Point, Dassen-, Dyer- and Robben Island), year (2016 and 2017) and host age (adult penguins and chicks) with body mass, body condition, haematocrit and total plasma protein at five African penguin colonies during the autumn/winter season. Significant values: ***=<0.001, **= 0.001-0.01, *=0.01-0.05, ·=non-significant.

Type of Analysis	Dependent variable	Intercept	Independent variables	Estimate	Standard error	p-value
On-host parasite abundance at all colonies (autumn/winter season)						
glm 'neg. bin.'	body mass		fleas	0.0008067	0.0024422	.
			ticks	-0.0148777	0.0103417	.
			lice	0.0926976	0.0620995	.
		Simon`s Town	Dassen Is.	0.0306275	0.0387691	.
		Simon`s Town	Dyer Is.	-0.0177764	0.0397086	.
		Simon`s Town	Robben Is.	0.0562066	0.0381931	.
		Simon`s Town	Stony P.	0.1122885	0.0342867	**
		year 2016	year 2017	0.0073764	0.0228589	.
		adult penguin	chick	-0.2948329	0.0274722	***
glm 'gaussian'	body condition		fleas	0.001441	0.003525	.
			ticks	-0.020998	0.014248	.
			lice	0.078539	0.085341	.
		Simon`s Town	Dassen Is.	-0.208049	0.065576	**
		Simon`s Town	Dyer Is.	-0.079062	0.067492	.
		Simon`s Town	Robben Is.	-0.004997	0.063995	.
		Simon`s Town	Stony P.	-0.00985	0.055795	.
		year 2016	year 2017	-0.045116	0.037695	.
glm 'poisson'	haematocrit		fleas	-0.0013695	0.001499	.

			ticks	-0.0001261	0.0060853	.
			lice	-0.0412952	0.0380861	.
		Simon`s Town	Dassen Is.	0.1921658	0.0266443	***
		Simon`s Town	Dyer Is.	0.0837569	0.0225465	***
		Simon`s Town	Robben Is.	0.0892177	0.021738	***
		Simon`s Town	Stony P.	0.0242029	0.0200735	.
		year 2016	year 2017	-0.016978	0.0143004	.
		adult penguin	chick	-0.3894202	0.0158377	***
glm 'gaussian'	total plasma protein		fleas	0.010123	0.006184	.
			ticks	0.011652	0.02577	.
			lice	-0.137454	0.153382	.
		Simon`s Town	Dassen Is.	0.172686	0.121049	.
		Simon`s Town	Dyer Is.	0.166143	0.099086	.
		Simon`s Town	Robben Is.	0.076594	0.094787	.
		Simon`s Town	Stony P.	-0.002073	0.085297	.
		year 2016	year 2017	-1.696371	0.062123	***
		adult penguin	chick	-1.306731	0.072494	***
Parasite richness at all colonies (autumn/winter season)						
glm 'neg. bin.'	body mass		ectoparasites	-0.05138	0.02293	*
			haemoparasites	0.04554	0.02774	.
		Simon`s Town	Dassen Is.	0.01962	0.04031	.
		Simon`s Town	Dyer Is.	-0.03881	0.04078	.
		Simon`s Town	Robben Is.	0.04724	0.03993	.
		Simon`s Town	Stony P.	0.08803	0.03542	*
		year 2016	year 2017	0.01162	0.02307	.

		adult penguin	chick	-0.27066	0.0292	***
glm 'neg. bin.'	body mass		helminth parasites	0.08144	0.02695	**
		Simon`s Town	Dassen Is.	0.11439	0.05956	.
		Simon`s Town	Dyer Is.	-0.03277	0.05967	.
		Simon`s Town	Robben Is.	0.16295	0.05992	**
		Simon`s Town	Stony P.	0.16654	0.04971	***
		year 2016	year 2017	0.07741	0.03504	*
glm 'gaussian'	body condition		ectoparasites	-0.062529	0.044276	.
			helminth parasites	0.010544	0.035738	.
			haemoparasites	-0.038646	0.049451	.
		Simon`s Town	Dassen Is.	-0.276183	0.083999	**
		Simon`s Town	Dyer Is.	-0.096849	0.083984	.
		Simon`s Town	Robben Is.	-0.011693	0.08381	.
		Simon`s Town	Stony P.	-0.013576	0.069956	.
		year 2016	year 2017	-0.005561	0.047985	.
glm 'poisson'	haematocrit		ectoparasites	-0.04441	0.014	**
			haemoparasites	-0.0617	0.01705	***
		Simon`s Town	Dassen Is.	0.16369	0.0275	***
		Simon`s Town	Dyer Is.	0.06071	0.02359	*
		Simon`s Town	Robben Is.	0.0661	0.02311	**
		Simon`s Town	Stony P.	0.03259	0.02109	.
		year 2016	year 2017	-0.01188	0.01484	.
		adult penguin	chick	-0.35427	0.0171	***
glm 'poisson'	haematocrit		helminth parasites	0.03484	0.01552	*
		Simon`s Town	Dassen Is.	0.23631	0.04097	***

		Simon`s Town	Dyer Is.	0.07848	0.03407	*
		Simon`s Town	Robben Is.	0.0973	0.03421	**
		Simon`s Town	Stony P.	0.0222	0.02881	.
		year 2016	year 2017	-0.01236	0.02182	.
glm 'gaussian'	total plasma protein		ectoparasites	0.05949	0.06025	.
			haemoparasites	0.08907	0.07093	.
		Simon`s Town	Dassen Is.	0.16708	0.12388	.
		Simon`s Town	Dyer Is.	0.15765	0.10237	.
		Simon`s Town	Robben Is.	0.07142	0.1007	.
		Simon`s Town	Stony P.	-0.05215	0.08932	.
		year 2016	year 2017	-1.68673	0.06396	***
		adult penguin	chick	-1.27818	0.07773	***
glm 'gaussian'	total plasma protein		helminth parasites	0.0219	0.05705	.
		Simon`s Town	Dassen Is.	0.2165	0.15367	.
		Simon`s Town	Dyer Is.	-0.05187	0.12281	.
		Simon`s Town	Robben Is.	0.08889	0.12327	.
		Simon`s Town	Stony P.	0.03906	0.10185	.
		year 2016	year 2017	-1.66342	0.07827	***

Table 3.4. Relationship between on-host parasite abundance (fleas, ticks and lice), parasite richness (ecto-, haemo, and helminth parasites species), season (autumn/winter and spring), host age (adult penguins and chicks) and year (2016 and 2017) with body mass, body condition, haematocrit and total plasma protein at the Stony Point African penguin colony. Significant values: ***=<0.001, **=0.001-0.01, *=0.01-0.05, ·=non-significant.

Type of Analysis	Dependent variable	Intercept	Independent variables	Estimate	Standard error	p-value
Parasite abundance at Stony Point (autumn/winter and spring season)						
glm 'neg. bin.'	body mass		fleas	-0.002991	0.00293	.
			ticks	-0.00563	0.007022	.
			lice	0.068825	0.197791	.
		autumn/winter	spring	-0.474478	0.044228	***
		adult penguin	chick	-0.314011	0.045733	***
		year 2016	year 2017	-0.113297	0.039	**
glm 'gaussian'	body condition		fleas	-0.001262	0.004689	.
			ticks	-0.017971	0.011229	.
			lice	0.178933	0.313222	.
		autumn/winter	spring	-0.637311	0.075913	***
		year 2016	year 2017	-0.007981	0.0695	.
glm 'poisson'	haematocrit		fleas	-0.001368	0.002113	.
			ticks	-0.01118	0.005489	*
			lice	-0.185072	0.140121	.
		autumn/winter	spring	-0.231443	0.031879	***
		adult penguin	chick	-0.437055	0.027374	***
		year 2016	year 2017	-0.035564	0.024648	.
glm 'gaussian'	total plasma protein		fleas	-0.0001602	0.0073985	.
			ticks	-0.006102	0.0176151	.

			lice	-0.2653773	0.4791271	.
		autumn/winter	spring	-2.0829064	0.1146412	***
		adult penguin	chick	-1.4271475	0.1154718	***
		year 2016	year 2017	-1.7152329	0.0960355	***
Parasite richness at Stony Point (autumn/winter and spring season)						
glm 'neg. bin.'	body mass		ectoparasites	-0.05392	0.02845	.
			haemoparasites	0.05389	0.03237	.
		autumn/winter	spring	-0.4524	0.04414	***
		adult penguin	chick	-0.33677	0.04926	***
		year 2016	year 2017	-0.10669	0.03859	**
glm 'neg. bin.'	body mass		helminth parasites	0.03629	0.02961	.
		autumn/winter	spring	-0.54335	0.05342	***
		year 2016	year 2017	-0.12038	0.0498	*
glm 'gaussian'	body condition		ectoparasites	-0.02561	0.05128	.
			helminth parasites	0.01585	0.03864	.
			haemoparasites	0.02527	0.05231	.
		autumn/winter	spring	-0.63561	0.08209	***
		year 2016	year 2017	0.01472	0.06516	.
glm 'poisson'	haematocrit		ectoparasites	-0.04324	0.01945	*
			haemoparasites	-0.06134	0.02247	**
		autumn/winter	spring	-0.23059	0.03103	***
		adult penguin	chick	-0.39621	0.03019	***
		year 2016	year 2017	-0.04008	0.02482	.
glm 'poisson'	haematocrit		helminth parasites	0.01743	0.02005	.
		autumn/winter	spring	-0.328	0.039	***

		year 2016	year 2017	-0.04571	0.03157	.
glm 'gaussian'	total plasma protein		ectoparasites	0.05452	0.07243	.
			haemoparasites	-0.12364	0.08135	.
		autumn/winter	spring	-2.13984	0.11266	***
		adult penguin	chick	-1.35801	0.12568	***
		year 2016	year 2017	-1.75715	0.09607	***
glm 'gaussian'	total plasma protein		helminth parasites	-0.03518	0.06667	.
		autumn/winter	spring	-2.21402	0.12335	***
		year 2016	year 2017	-1.72981	0.10956	***

Discussion

Clinical parameters of African penguins along the south-western coast

It is evident from the data that there is intraspecific variation in the clinical parameters between colonies and in some cases the recorded values for the parameters differed from the reference range for healthy penguins. Between colonies, most of the mean body mass of adult penguins and chicks were lower than the reference mean values recorded for healthy adult African penguins and chicks (Parsons et al. 2015, 2016). These differences can be due to a much larger sample size and different sampling period in the present study (201 adults and 476 chicks sampled in 2 years compared to 42 adults and 30 chicks sampled in the Western Cape during 3-4 years in Parsons et al. 2015, 2016). Body mass is related to food intake (Brown and Sherry 2006). During the breeding season adult penguins are range restricted, being central place foragers, returning regularly to the colony to feed their chicks (Clarke 2001, Crawford et al. 2006b, Boersma 2008). It is therefore inferred that colonies with adults and chicks that exhibited lower body mass are probably areas with limited food resources. Adult penguins at Dassen and Robben Island recorded a lower mean body mass compared to the other colonies, which may be due to reduced food availability in those areas as a result of the eastward displacement of fish and fishing pressure (Van der Lingen et al. 2015, Grémillet et al. 2008, Coetzee et al. 2008, Sherley et al. 2018). Chicks depend on the parents to obtain their food and gain body mass (Ricklefs 1987), and parents can regulate the meal provision to chicks according to their own body condition which would suggest that the parents' body condition can also play a role in chick body mass (Tveraa et al. 1998). Chicks from Dassen and Robben Island did not follow the adult pattern, which may be a result of the effort by the parents to feed chicks in an area with reduced food resources. Conversely, chicks at Simon`s Town and Dyer Island may not be receiving enough food from their parents compared to Stony Point. Another factor that can play a role in the body mass of penguin chicks is the age of the chicks. We are unsure about the exact age of the chicks sampled in the study by Parsons et al. (2016). Therefore, it is also possible that a large proportion of those chicks were older (larger) than the ones we sampled, increasing the mean value of reference. Adult penguin and chick body mass were significantly reduced in spring (warmer and drier season) compared to autumn/winter (cold

and rainy season) at Stony Point. Higher temperatures (such as those in spring) can induce loss of water and thus affect their mass (McLean et al. 2018). Also, it has been suggested that a heavier body mass during the winter is associated to higher body fat reserved in order to cope with the adverse cold conditions, which in turn might not be as required during warmer seasons (Lima 1986). For example, king penguins (*Aptenodytes patagonicus*) increase their body fat (in chicks), and protein, water and fat (in adults) content at the beginning of winter to cope with the consecutive fasting period (Cherel et al. 1993). Previous studies have also reported body mass loss in bird species as they move from cold and rainy seasons/years to hotter and drier ones (Yom-Tov 2001, Brown and Sherry 2006, Yom-Tov et al. 2006, Van Buskirk et al. 2010, Salewski et al. 2010). Climatic factors of seasons such as temperature and rainfall can influence the abundance of food resources, which in turn will affect bird body mass (Brown and Sherry 2006, Yom-Tov et al. 2006). In South Africa, the most common prey of African penguins (sardine *Sardinops sagax* and anchovy *Engraulis encrasicolus*) (Crawford et al. 2011) experience a seasonal migration from the west to the south coast (Agulhas Bank) from August-September until March-April in order to find spawning fields (Crawford 1980). Therefore, part of the diet consumed by penguins is greatly reduced in the spring-summer season.

The mean body condition of chicks across colonies fell within the normal range established for healthy African penguin chicks (Lubbe et al. 2014). However, chicks at Dassen Island recorded a poorer body condition compared to the other colonies throughout the study period. Apart from parasite infestations, body condition can be influenced by food availability (Brown 1996, Lubbe et al. 2014), brood size (Roulin et al. 2003), colony size, crowding within a colony, food competition with neighbouring seabird colonies (Tella et al. 2001) and parental supplementation of chicks (Ricklef et al. 1985, Bolton 1995, Weimerskirch 1998, O'Dwyer et al. 2006). Although no significant relationship was recorded for on-host parasite infestations on chick body condition, there are other possible factors that may be responsible for the lower body condition of chicks at Dassen Island. Dassen Island is one of the most populated colonies along the south-western coast of South Africa (1862 and 1922 breeding pairs in 2016 and 2017, respectively; Department of Environmental Affairs and SANParks upublished data). Given the fact that penguins are

central place foragers this could lead to competition for limited food resources in the area (due to the eastward displacement of fish). Campbell (2016) found that the body condition African penguin chicks at Robben Island significantly increased with the abundance of forage fish around the island, suggesting that the lower body condition of chicks observed at Dassen Island could be food related. Furthermore, Sherley et al. (2018) showed that fishing closures improved chick condition after controlling for prey availability and so both studies indicated that abundance of prey within the core foraging range of a central place forager has an impact on offspring fitness. A large number of breeding penguins on Dassen Island can also lead to over-crowing and increased stress in the colony. For example, a study on Magellanic penguins (*Spheniscus magellanicus*) recorded a lower body condition for fledglings in areas of high nest and bird density, possibly because of existing competition for food resources in the area (Tella et al. 2001). Further, in other seabirds such as sooty shearwater (*Puffinus griseus*) and Gould's petrels (*Pterodroma leucoptera*), the decision of parents to feed their chicks seems to be influenced by chick nutritional requirements (O'Dwyer et al. 2006) and by intrinsic factors of the parents such as adult body mass (Weimerskirch 1998). It is interesting to note that in the present study the average body mass of adult penguins at Dassen Island was one of the lowest values. Seasonal variation in the body condition of chicks was recorded at Stony Point. During autumn/winter 2016 most of the chicks recorded body condition values within the normal range described for African penguin chicks (Lubbe et al. 2014). While during spring 2016 most values were below the reference range. This result was also confirmed by the regression models, where chicks exhibited a significantly lower (poorer) body condition during spring compared to autumn/winter in 2016. Climatic conditions not only exerts a direct effect on a bird's physiology (e.g. affecting thermoregulation), but also affects prey abundance (Brown and Sherry 2006, Descamps et al. 2010). As mentioned above, the common prey species consumed by African penguins migrate towards the east coast during the warmer months of the year (Crawford 1980). In addition, most African penguins moult between September and January in South Africa (Crawford et al. 2006c). During moulting, adult penguins leave the colony for a ~ 4 week period to fatten-up (build sufficient fat reserves) to survive the subsequent starvation period on land (Randall and Randall 1981,

Crawford et al. 2006c). During this time, any chicks that have not fledged are mostly unattended and not fed (Sherley et al. 2014). These factors can contribute to the poor body condition of chicks found at Stony Point during the spring season. In colonial birds, such as the great tit (*Parus major*), a similar pattern in seasonal variation was recorded with a higher body condition index during autumn and winter compared to spring (Norte et al. 2009). Further, during spring several morphological parameters such as fat and muscle score also decreased (Norte et al. 2009). In seabirds, such as the black-legged kittiwakes (*Rissa tridactyla*), seasonal decline in body condition was more severe in birds from food-restricted colonies compared to food-abundant colonies (Kitaysky et al. 1999).

Between colonies, haematocrit values recorded from adult penguins and chicks showed values mostly within the normal reference range for African penguin adults (Parsons et al. 2015) and chicks (Parsons et al. 2016). Nevertheless, haematocrit from all penguins were higher at island colonies (Dassen, Dyer and Robben Island) compared to those from penguins at mainland colonies (Simon's Town and Stony Point). Some contributing factors to the lower haematocrit include nutritional deficiencies and chronic infection (e.g. parasitism) (Samour 2006, Campbell and Ellis 2007). Haematocrit differences between geographically close sites have also been reported for wild little penguins (Sergent et al. 2004). As in our study, little penguins have shown lower haematocrit on locations that were close to highly urbanized areas, suggesting that stress factors present near anthropogenic influence can affect health parameters of penguins. In particular, chicks at Dassen Island showed higher haematocrit than chicks at all other colonies. These differences suggest that, as for body condition, chicks at Dassen might be receiving little fluids from their parents and may be slightly dehydrated compared to the other four colonies in the study. Season showed a significant effect on the penguin haematocrit at Stony Point. Haematocrit vales tended to be lower in spring compared to autumn/winter. Season is considered an important factor affecting haematological parameters in birds (Norte et al. 2009). The pattern recorded in the present study is consistent with the findings of other authors who have also recorded a lower haematocrit in birds during warmer seasons of the year (reviewed by Fair et al. 2007). These differences have been attributed to increase metabolic rate to satisfy demands of thermogenesis, moulting and reproductive stage (Fair et al. 2007, Norte et al.

2009). Poor body condition of birds has also been indicated as a factor associated with low haematocrit (Sergent et al. 2004). In our study, chicks were in poor body condition during spring. The low availability of food resources (fish) along the west coast during spring months (Crawford 1980), and the resulting nutritional deficiencies could be reflected in a low erythrocytes production (Campbell and Ellis 2007). This has also been reported in chinstrap penguins and little penguins (Merino and Barbosa 1997, Sergent et al. 2004).

In this study, the TPP from adult penguins and chicks across colonies were higher than the total serum protein values recorded for healthy wild African penguins. A higher value of proteins in plasma compared to those in serum is anticipated since plasma proteins include clotting factors (e.g. fibrinogen) which are absent in serum (Andreasen et al. 1989). However, the total protein should only be approximately 0.1g/dl higher (Lumeij 1987). Previous studies on captive African penguin adults reported a TPP of 6.3g/dl (±1.4) (Cray et al. 2010), which is comparable to the serum protein values (5.9g/dl ±0.96) recorded by Parsons et al. (2015) for healthy wild adult African penguin from the Eastern and Western Cape in South Africa. A possible reason for the differences observed could be due to the methods used to determine TPP. We used a hand-held refractometer, which is an instrument that uses the refraction of the light through aqueous substances to provide a simple and fast estimation of solutes in fluids. All solids in solutions can contribute to an angle of refraction, including protein (i.e. albumin, globulin and fibrinogen) and non-protein substances (e.g. glucose, sodium, chloride, phosphate, urea and lipids) (George 2001, Briend-Marchal et al. 2005). Therefore, a variation on the non-protein components can alter the solution density and cause false high protein estimation (George 2001, Hayes et al. 2011, Hunsaker et al. 2016). Some authors have obtained higher results using refractometers than more sensitive methods (e.g. colorimetric biuret) especially in avian species (Lumeij and De Bruijne 1985, Lumeij 1987, Newman et al. 1997, Campbell and Ellis 2007). Moreover, the values of total serum protein used as reference to compare our results were obtained using a colorimetric method (Parsons et al. 2015), which could also explain differences from the values that we obtained. In addition, the reference values for serum protein were obtained from African penguins sampled between 2010 and 2013, while our samples were collected in 2016 and 2017. In the event that the increase in TPP

observed in our study is true, then this could be due to an increase in dietary protein intake, dehydration and acute or chronic infection or inflammatory conditions with a decrease in album:globulin ratio (Lumeij 1987, Gregg et al. 2006). Differences in TPP between colonies were also recorded, where lower mean TPP was recorded from penguins at Dassen Island. A decrease in TPP could be due to nutritional deficiencies, starvation and acute ectoparasite infestations (Ots et al. 1998, Quillfeldt et al. 2004). The poor body condition of chicks on Dassen Island discussed above, potentially associated to low availability of food resources in the area, might explain the low TPP recorded from penguins in this island. Season of the year showed a significant effect at Stony Point, where TPP was lower in penguins during spring compared to autumn/winter. A reasonable explanation is the poor body condition exhibited by most of the chicks during spring compared to winter due to, as mentioned before, a possible food restriction during spring in South Africa. As discussed, a lower intake of food resources and a poor nutrition are causes of TPP decrease; hence, a seasonal effect on TPP is not unexpected.

Relationship between on-host parasites and clinical values of penguins

The parasite species richness of African penguins across colonies potentially affected several of the clinical parameters. In general, ectoparasite species richness had a negative relationship with body mass of African penguins. This pattern is supported by previous studies. In particular, Hughes and Page (2007) recorded a negative effect of lice species richness and the body mass of seabirds (Charadriiformes, Pelecaniformes and Procellariiformes). In addition, Norte et al. (2013) argued that the anemia and tissue damage produced by ectoparasites stimulate an increase in resource consumption through metabolic processes, resulting in a decrease in the host's body mass. Ecto- and haemoparasite species richness also had a negative effect on the haematocrit of African penguins in the present study. Most of the ectoparasites in this study included blood sucking arthropod species (the fleas *P. humboldti* and *E. gallinacea*, and the soft tick *O. capensis*), while haemoparasites belonged to the orders Piroplasmorida/Haemospororida which comprises protozoan parasites such as *Babesia* spp., *Plasmodium* spp., and *Leucocytozoon* spp., and the order Spirochaetales which comprises the bacterial parasite

Borrelia spp. All of these parasites can cause anemia (decrease haematocrit) in birds through blood loss (ectoparasites) and haemolysis (haemoparasites) (Samour 2006, Campbell and Ellis 2007). A similar pattern was recorded for wild Magellanic penguins that were exposed to various ectoparasites (Hawkey et al. 1989).

In contrast, helminth species richness had a positive relationship with the body mass in African penguins. This relationship may be explained by the diet of penguins. Since African penguin acquire helminth parasites such as *Cardiocephaloides* spp., *Renicola* spp., *Contracaecum* spp. and *Cyathostoma* spp. through their fish diet (Horne et al. 2011, Reed et al. 2012, Kanarek et al. 2013) it can been inferred that higher fish consumption will expose penguins to more helminths and a larger number of helminth species. The consumption of fish can also facilitate an increase in haematocrit values in penguins. An increase in food intake (reflected in good nutritional condition) could increase the proportion of red cells in blood (Piersma et al. 2000, Fair et al. 2007). This is supported by Hoi-Leitner et al. (2001), who found a positive relationship between the haematocrit values of serin (*Serinus serinus*) nestlings and food availability.

On-host tick abundance and parasite richness were important predictors of clinical parameters of African penguins sampled during the autumn/winter and spring seasons at Stony Point. In particular, tick abundance was inversely related to haematocrit of penguins in this study. The examination of tick abundance across colonies (Table 3.2) showed differences between Stony Point and most of the other colonies, suggesting that conditions at Stony Point might be promoting a greater abundance of ticks. In addition, the negative relationship between ectoparasite richness and penguin's haematocrit, and haemoparasite richness and penguin's haematocrit could be driven by the warmer season (spring) in Stony Point. A higher prevalence arthropod vector is reported when temperatures increase, which in turn can facilitate the transmission of haemoparasites (Quillfeldt et al. 2011, Yabsley et al. 2012). In addition, given that most of the chicks exhibited a poor body condition during the spring season, it is expected that parasites increase their colonization (De Lope et al. 1993).

Conclusions

The present study provides valuable data on the general health status of wild African penguins along the south-western coast of South Africa and highlight potential factors that may influence clinical parameters across penguin colonies. Although most of the clinical parameters exhibited mean values considered as normal for the species, there is between colony variations. Important factors that might have caused variation on clinical parameters of penguins included the availability of food, season of the year and parasite richness. In particular, the study provides evidence that ecto- and haemoparasite species richness could be exerting negative effects on the health of African penguins. The extent of the effect of parasites on penguin health will vary between colonies as each colony has a set of unique characteristics that can either exacerbate or mitigate the conditions for parasite infestation and the potential effect on penguin health.

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Supplementary material

Table S3.1. Model selection based on Akaike Information Criterion (AIC) across colonies. Numbers in brackets represent the different scenarios derived from the full model (assessed in the study) to reach the best model (in bold). Significant differences between the full and the best model (p-value) according to Chi-square test. Independent variables using parasite abundance: fleas, ticks, lice, colony (Stony Point, Simon's Town, Dassen-, Dyer- and Robben Island), year (2016 and 2017), age (adult and chick). Independent variables using parasite richness: ectoparasites (fleas, ticks and lice), haemoparasites (Piroplasmorida/Haemosporida and Spirochaetales), helminth parasites (*Cardiocephaloides* spp., *Renicola* spp., *Contracaecum* spp. and *Cyathostoma* spp.), colony (Stony Point, Simon's Town, Dassen-, Dyer- and Robben Island), year (2016 and 2017) and age (adult and chick).

Model	AIC	Chisq	df	p-value
On-host parasite abundance at all colonies (autumn/winter season)				
BODY MASS				
(1) body mass ~ fleas + ticks + lice + colony + year + age	10839.42			
(2) body mass ~ fleas + ticks + lice + colony + age	10837.52			
(3) body mass ~ ticks + lice + colony + age	10835.68	3.74	2	>0.05
BODY CONDITION				
(1) condition ~ fleas + ticks + lice + colony + Year	506.48			
(2) condition ~ ticks + lice + colony + Year	504.65			
(3) condition ~ ticks + colony + Year	503.52			
(4) condition ~ ticks + colony	502.69	3.79	3	>0.05
HAEMATOCRIT				
(1) haematocrit ~ fleas + ticks + lice + colony + year + age	3820.91			
(2) haematocrit ~ fleas + lice + colony + year + age	3818.91			
(3) haematocrit ~ lice + colony + year + age	3817.75			

(4) haematocrit ~ colony + year + age	3816.98			
(5) haematocrit ~ colony + age	3816.65	4.26	4	>0.05
TOTAL PLASMA PROTEIN				
(1) total plasma protein ~ fleas + ticks + lice + colony + year + age	1349.26			
(2) total plasma protein ~ fleas + ticks + lice + year + age	1347.09			
(3) total plasma protein ~ fleas + lice + year + age	1345.23			
(4) total plasma protein ~ fleas + year + age	1343.74			
(5) total plasma protein ~ year + age	1343.13	6.13	4	>0.05
Parasite richness at all colonies (autumn/winter season)				
BODY MASS				
(1) body mass ~ ectoparasites + haemoparasites + colony + year + age	10021.57			
(2) body mass ~ ectoparasites + haemoparasites + colony + age	10019.82	1.75	1	>0.05
(1) body mass ~ helminth parasites + colony + year	5760.15	0	NA	NA
BODY CONDITION				
(1) condition ~ ectoparasites + helminth parasites + haemoparasites + colony + year	355.99			
(2) condition ~ ectoparasites + helminth parasites + haemoparasites + colony	354.01			
(3) condition ~ ectoparasites + haemoparasites + colony	352.13			
(4) condition ~ ectoparasites + colony	350.77	5.22	3	>0.05
HAEMATOCRIT				
(1) haematocrit ~ ectoparasites + haemoparasites + colony + year + age	3580.54			
(2) haematocrit ~ ectoparasites + haemoparasites + colony + age	3579.19	1.35	1	>0.05
(1) haematocrit ~ helminth parasites + colony + Year	2036.19			
(2) haematocrit ~ helminth parasites + colony	2034.51	1.68	1	>0.05
TOTAL PLASMA PROTEIN				
(1) total plasma protein ~ ectoparasites + haemoparasites + colony + year + age	1260.94			

(2) total plasma protein ~ ectoparasites + haemoparasites + year + age	1259.91			
(3) total plasma protein ~ haemoparasites + year + age	1258.08			
(4) total plasma protein ~ year + age	1256.28	4.66	3	>0.05
(1) total plasma protein ~ helminth parasites + colony + year	651.37			
(2) total plasma protein ~ helminth parasites + year	646.97			
(3) total plasma protein ~ year	645.11	6.26	2	<0.05

Table S3.2. Model selection based on Akaike Information Criterion (AIC) at Stony Point. Numbers in brackets represent the different scenarios derived from the full model (assessed in the study) to reach the best model (in bold). Significant differences between the full and the best model (p-value) according to Chi-square test. Independent variables using parasite abundance: fleas, ticks, lice, season (autumn/winter and spring season), age (adult and chick) and year (2016 and 2017). Independent variables using parasite richness: ectoparasites (fleas, ticks and lice), haemoparasites (Piroplasmorida/Haemospororida and Spirochaetales), helminth parasites (*Cardiocephaloides* spp., *Renicola* spp., *Contracaecum* spp. and *Cyathostoma* spp.), year (2016 and 2017), age (adult and chick) and season (autumn/winter and spring season).

Model	AIC	Chisq	df	p-value
Parasite abundance at Stony Point (autumn/winter and spring season)				
Y= BODY MASS				
(1) body mass ~ fleas + ticks + lice + season + age + year	4799.58			
(2) body mass ~ fleas + ticks + season + age + year	4797.71			
(3) body mass ~ fleas + season + age + year	4796.36			
(4) body mass ~ season + age + year	4795.2	4.38	3	>0.05
Y= BODY CONDITION				
(1) condition ~ fleas + ticks + lice + season + year	316.5			
(2) condition ~ fleas + ticks + lice + season	314.51			

(3) condition ~ ticks + lice + season	312.6			
(4) condition ~ ticks + season	310.97	5.53	3	>0.05
Y= HAEMATOCRIT				
(1) haematocrit ~ fleas + ticks + lice + season + age + year	1817.49			
(2) haematocrit ~ ticks + lice + season + age + year	1815.91			
(3) haematocrit ~ ticks + lice + season + age + year	1815.67	1.82	2	>0.05
Y= TOTAL PLASMA PROTEIN				
(1) total plasma protein ~ fleas + ticks + lice + season + age + year	586.62			
(2) total plasma protein ~ ticks + lice + season + age + year	584.62			
(3) total plasma protein ~ lice + season + age + year	582.75			
(4) total plasma protein ~ season + age + year	581.06	5.56	3	>0.05
Parasite richness at Stony Point (autumn/winter and spring season)				
Y= BODY MASS				
(1) body mass ~ ectoparasites+ haemoparasites+ year + age + season	4577.12	0	NA	NA
(1) body mass ~ helminth parasites + year + season	2946.53			
(2) body mass ~ year + season	2946.03	0.5	1	>0.05
Y= BODY CONDITION				
(1) condition ~ ectoparasites+ helminth parasites + haemoparasites+ season + year	162.72			
(2) condition ~ ectoparasites+ helminth parasites + haemoparasites+ season	160.77			
(3) condition ~ ectoparasites+ haemoparasites+ season	158.91			
(4) condition ~ ectoparasites+ season	157.12			
(5) condition ~ season	155.4	7.32	4	>0.05
Y= HAEMATOCRIT				
(1) haematocrit ~ ectoparasites+ haemoparasites+ year + age + season	1786.94	0	NA	NA
(1) haematocrit ~ helminth parasites + year + season	1128.75			

(2) haematocrit ~ year + season	1127.51	1.24	1	>0.05
Y= TOTAL PLASMA PROTEIN				
(1) total plasma protein ~ ectoparasites+ haemoparasites+ year + Age + season	574.46			
(2) total plasma protein ~ haemoparasites+ year + age + season	573.03	1.43	1	>0.05
(1) total plasma protein ~ helminth parasites + year + season	346.93			
(2) total plasma protein ~ year + Season	345.22	1.71	1	>0.05

Chapter 4

Relationship between nest characteristics and nest ectoparasite infestations and the potential effect on the general health of African penguins

(Prepared for *Parasitology*)

Abstract

Bird nests can provide a suitable microclimate for the development of nest-dwelling ectoparasites. Little is known with regard to the microclimatic conditions associated with African penguin nests and the impact on ectoparasite infestations. The aims of the study were to assess the effect of various nest characteristics on the abundance of nest ectoparasites and the health of African penguins in the Stony Point colony, South Africa. Penguins (n=50 adults and 192 chicks) and their nests (n=308) were sampled seasonally during 2016 and once in 2017. Ectoparasites were recorded from penguins and their nests. Blood was collected for haematological analyses and haemoparasites incidence. Microclimatic conditions in nests were assessed with iButtons and soil samples. Several nest characteristics were recorded. Ectoparasite infestations were higher under warmer and drier conditions, in artificial nests and nests near the coastline. Flea abundance was higher in warm and moist nests occupied by a bird. Penguin body mass was lower in natural open compared to other nest types. Ectoparasites benefit from microclimatic conditions associated with artificial and natural covered nests. Given the potential adverse effects of ectoparasites on penguin health, it is advisable that a parasite and penguin health monitoring programme must be initiated in the colony.

Key words: Penguin nest characteristics; nest microclimatic conditions; nest-dwelling ectoparasites; African penguin conservation.

Introduction

Nests represent an important component of bird ecology as it is the physical place where birds find protection from the external environment (e.g. weather and predators) and fulfil reproductive tasks (e.g. egg laying, egg incubation and chick rearing) (Mainwaring *et al.* 2014). Nest designs and types are diverse and include amongst others nests on the ground surface, in tree holes (cavities) or attached to walls (Hansell, 2000). The type of material used to build the nest is equally diverse and is generally sourced from the surrounding environment (Deeming and Mainwaring, 2015). Although it is not uncommon for a bird species to have one characteristic nest type, there are several species that are opportunistic and make use of several nest designs. However, not all nest types are equal as they differ in exposure to predators and climatic conditions which can impact the breeding success of the species (Mitrus, 2003; Freund *et al.* 2017). Bird nests also often provide ideal habitats for nidicolous invertebrates such as ectoparasites to develop and survive (Daturi, 1986; Marshall, 1981; López-Rull and Macías-Garcia, 2015).

Ectoparasites such as soft ticks, fleas and louse flies are closely associated with nests and or cracks and crevices around the nests where they find a sheltered place to live, breed and feed (Dobroscky, 1925; Fryderyk and Izdebska, 2009). Given their exothermic nature, it is expected that the prevailing microclimatic conditions associated with specific nest types will influence the abundance and prevalence of ectoparasites within nests. For example, the abundance of adult and larval stages of the flea *Ceratophyllus gallinae* was positively affected by the humidity associated with hole nests of passerine birds (Heeb *et al.* 2000). In addition, the incidence of fleas and soft ticks in nests were negatively affected by high moisture content in nests (Daturi, 1986; Heeb *et al.* 2000). Furthermore, studies on passerine birds have shown that artificial nest boxes have a higher prevalence of fleas than natural tree cavities, suggesting that the use of water-proof material in artificial nests provide a drier and more protected environment for ectoparasites (Hebda and Wesołowski, 2012). Other nest characteristics that can also influence parasite infestations include the nest opening (Daturi, 1986), spatial position and orientation (Marshall, 1981; Merino and Potti, 1996) and nest age (Brown and Brown, 1986; Mazgajski, 2007). With regard to the latter, older nests tend to accumulate more nest material content (i.e. dry grass, leaves, barks, branches and herbs) which can provide a more favourable habitat for ectoparasites to reproduce and survive for

extended periods even in the absence of the host (Brown and Brown, 1986; Mazgajski, 2007). Although not a physical attribute of the nest, the occupancy of the nest can also affect the presence and abundance of certain ectoparasites. Fleas for instance are prevalent and abundant when nests are occupied by incubating adults and chicks (Marshall, 1981), while nidicolous ticks remain in the nests regardless of the presence of a host (Sonenshine, 1993).

Nest ectoparasites can have detrimental effects on their bird host and these vary across seasons and years (Dobroscky, 1925; Mangin *et al.* 2003). Differences in ectoparasite abundance and prevalence in bird nests have been attributed to variation in weather conditions (e.g. temperature and rainfall) that facilitates the development of certain parasites and enhances their effects on their hosts in different seasons and years (Merino and Potti, 1996). Direct effects include hyper-infestation with the associated stress and anaemia (Lehmann, 1993), reduction in chick growth (Proctor and Owens, 2000) and a decline in survival of chicks and adults (Clarke and Kerry, 1993), decreased reproductive performance in breeding adults (Mangin *et al.* 2003), and inducing adult birds to abandon eggs and chicks (King *et al.* 1977; Duffy, 1983). Indirectly, nest ectoparasites can transmit a great diversity of pathogenic agents, such as viral, protozoan, spirochetal and rickettsial diseases that can cause severe reductions in host population size (Proctor and Owens, 2000; Fryderyk and Izdebska, 2009; Bitam, 2010). The presence of ectoparasites in nests is thus a subject of concern for conservationists who recognise them as a potential threat to endangered wild bird populations (Williams *et al.* 2013).

The African penguin (*Spheniscus demersus*) is an endangered seabird species that currently nests in 24 island and four mainland colonies distributed along the coast of Namibia and South Africa (Crawford *et al*. 2013). The historic collection of seabird guano from the islands is regarded as one of the factors that contributed to the dramatic population decline of the species (>50% in three generations) (BirdLife International, 2016). The removal of guano, the principal substrate used to build more protected burrows, brought about a change in nesting behaviour to the extent that a large portion of nests are more exposed open surface nests (Frost *et al.* 1976; Cooper, 1980). Presently, natural nests comprise surface nests that are open, holes and burrows (Frost *et al.* 1976). In addition, the use of artificial nests (made from cement, wood and fibreglass) was introduced in recent years in an attempt to improve the breeding success of the species at several colonies (Kemper *et al.* 2007; Sherley *et al.*

2012; Pichegru, 2013). Subsequent studies have however criticized the value of certain artificial nest types due to extreme ambient temperatures being recorded in fiberglass nests (Lei *et al.* 2014). African penguins use their nests to mate, incubate eggs and guard chicks. The breeding season in South Africa is extended across the year, usually from February to September/October (Cooper, 1980; Crawford *et al.* 2006). Adult penguins regularly lay two eggs that are incubated in the nest for approximately 40 days (37-38 days per egg) by both parents. After hatching the chick remains in the nests for approximately 80 days (this can range between 60-120 days) (Williams and Cooper, 1984), while the parents rotate to feed at sea during the day and return to the nest in the evening (Cooper, 1980).

The Stony Point penguin colony was established in Betty's Bay on the west coast of South Africa in 1982, following the arrival of a single penguin pair (Whittington *et al.* 1996). Since then the colony progressively grew in numbers to reach 2,533 breeding pairs by 2015 (CapeNature unpubl. data). The latter was most probably brought about by an increase in prey fish in the surrounding ocean (as a result of an eastward shift in fish stocks; Grémillet *et al.* 2008). Management interventions on the island include the use of artificial nests (cement and fibreglass) and reconditioned natural nests by covering them with vegetation in an attempt to provide more sheltered nests. Recently there were reports of nest and chick abandonment and managers observed soft ticks on chicks and in nests at the start of summer (warm-dry season). This has raised concerns that parasites and tick-transmitted pathogens may pose an additional threat to the conservation efforts for the species. Given the diverse nature of the colony, where penguins breed in a variety of nest types, it was important to develop a better understanding of the factors that drive this pattern. The aims of the study were to determine the effect of various nest characteristics on in-nest ectoparasite abundance and prevalence and if a pattern was found, to establish the effect on the general health of and the incidence of tick-transmitted pathogens in penguins.

Materials and methods

Study site

This study was carried out in the Stony Point penguin colony (34.3741° S, 18.8917° E) at Betty`s Bay along the south coast of South Africa. Betty`s Bay falls within the winter rainfall region of South Africa with cold and wet conditions generally characteristic for the period

May through September. Penguin nests, occupied with a chick and adult penguins, were randomly selected and sampled during three sampling seasons: June/July (autumn/winter) 2016=109 nests, 22 adult penguins and 83 chicks, October/November (spring) 2016=81 nests, 8 adults and 24 chicks, and June/July (autumn/winter) 2017=118 nests, 20 adults and 85 chicks (Fig. 4.1). In each season, the nests comprised three different nest types: artificial (fibreglass or cement-fibre nests (ca. 10 % cement nests)) (Fig. 4.2A), natural covered (nests sheltered by vegetation, i.e. *Tetragonia fruticosa*) (Fig. 4.2B) and natural open (nests established on the ground surface, with or without a few dry branches covering on top) (Fig. 4.2C). A total of 84 penguins (adults and chicks) from artificial, 87 penguins from natural covered and 71 penguins from natural open nests were sampled across the seasons (Table 4.1). The nests were selected 30 days before the actual field session to enable the recording of soil temperature within each nest over a 30-day period.

Methods used in the field

Soil temperature in nests

iButton data loggers (Thermochron®; temperature range -40 +85°C and resolution 0.5°C) were inserted in the soil inside each nest at a 5 cm depth. Each iButton was set to record temperature at 60-minute intervals during the 30 days before nest and penguin sampling were conducted. iButtons were dipped in a rubber coating (PLASTI DIP®) (three coats per device) for waterproofing prior to deployment. This step was included in the second (October/November 2016) and third field season (June/July 2017) due to the failure (as a result of moisture) of some iButtons during the first field season (June/July 2016). Subsequently, each iButton was placed in a small plastic pipe (that was open on one side and with holes on the sides, for contact with the soil), adhered to the tip of a wooden stick and inserted in the nest (Fig. 4.2D, 4.2E). This procedure was followed to facilitate recovery of the iButtons after 30 days and to prevent tampering by the penguins. All procedures were tested in the laboratory before the study commenced. The data from the iButtons were used to calculate the mean temperature and standard deviation (SD) from the values recorded over the 30-day period.

Samples collected from penguin nests

A standardised 200 ml sample was collected using a spade from the centre of each nest for the extraction of ectoparasites (ticks and fleas). The sample, which consisted of nest material (e.g. sticks, seaweed and stones) and soil, was transferred as is to a plastic jar and sealed with a lid. In addition, a 100 ml nest material sample (from the top layer, maximum depth 2 cm) and 50 ml soil sample (beneath the nest material) were collected from each nest. Both samples were individually placed in pre-weighed glass sample bottles and sealed with a lid. The latter samples were used to assess the moisture content of the nest material and soil in the nests. To limit any variation in collection method, the nest samples were collected by the same individual throughout the study.

Nest occupancy and age

Nest content was classified as "active" if there was an egg, chick, adult or a combination of them inside the nests, or "inactive" in the absence of any content. The age of each nest was obtained from colony records. Nests were characterised as: "1" - new nests or established within a year, "2" - nests established more than one and less than three years ago, and "3" - nests established more than three years ago.

Distance of nest from the coast

In Betty's Bay, the north-west winds predominate in winter and south-east winds predominate in spring (Barwell, 2015). Since Stony Point receives predominant winds from two different directions during the sampled seasons, we recorded the distance of each nest to the south-east coast and to the west coast of the colony. The geographic location of each nest was referenced using a GPS and the coordinates were used to calculate the distance (meters) drawing a straight line from each nest to the south-east and west coast using the measure tool of Google Earth Pro (image date 30/12/2017 ©2018 DigitalGlobe). In each case the direction of the lines were in the same direction and parallel to each other. This will give us an indication of the exposure to wind and moisture from sea sprays for each nest (Monahan, 1968).

Orientation and opening of nest entrance

The orientation of the nest entrance was recorded for artificial and natural covered nests only (natural open nests do not have a specific direction). Nests entrances were denoted as 'windward' or 'leeward' pending the predominant wind direction ('windward' in winter north-east and spring south-east and 'leeward' the nests facing the remaining directions). The size of the nest entrance was recorded horizontally from side-to-side at the widest point from artificial and natural covered nests using a measuring tape.

Samples and data collected from penguins

At each of the selected nests the adult penguin and chick were handled for ca. 8 minutes. Ectoparasites were collected from the abdominal area by brushing the plumage for 1 minute with a soft brush and from around the eyes using a forceps. The body regions were selected based on their association with ectoparasites from previous studies (Nola Parsons pers. comm. 2016). In order to assess penguin health various parameters (body mass, chick body condition, haematocrit and total plasma protein) we recorded. Penguin body mass (kg) was recorded using a handheld electronic scale (25kg/50lb Sensation) and head length (mm) was measured from the back of the head to the tip of the beak (Lubbe *et al.* 2014) using an electronic calliper (Grip 150mm Digital Vernier). Blood was collected from the dorsal aspect of the foot using a 23-gauge needle, and placed in 80 ul heparinized microhematocrit capillary tubes. Capillary tubes were centrifuged (on site and on the same day) using a portable centrifuge (Hawksley & sons Ltd.) at 14000 rpm for 5 minutes (Travis *et al.* 2006). Haematocrit (packed cell volume) was measured using a microhematocrit reader (Hawksley & sons Ltd.) and total plasma protein (TPP) was determined using a handheld refractometer calibrated before use (Bellingham and Stanley Ltd and Schmidt + Haensch). A drop of blood was also collected to make thin blood smears which were fixed with methanol on site to later assess the presence of tick-transmitted pathogens (haemoparasites).

Laboratory analysis

Nest ectoparasite extraction

Each nest sample was divided into two portions (100ml each) and placed on a grid inside a modified Berlese funnel (Southwood, 1978). A cloth bag containing 100 g of naphthalene

mothballs was suspended in the funnel above the sample, sealed with a plastic cover and left for 24 hours (Daturi, 1986). Each sample was subsequently systematically inspected using a stereomicroscope (Leica Microsystems, Wetzlar, Germany) to ensure the complete removal of ectoparasites from the nest sample. Ectoparasites were counted and identified morphologically to species level using taxonomic reference keys (Bedford, 1934; Jordan, 1942; Arthur, 1963; Kohls *et al.* 1965; Segerman, 1995). Thin blood smears were stained with Eosin-Methylene Blue stain (RapiDiff kit). Presence of haemoparasites was recorded in 150 fields per slide under a light microscope (Leica Microsystems, Wetzlar, Germany) at 100x magnification (Palinauskas *et al.* 2008; Valkiūnas *et al.* 2008). Haemoparasites were identified to order level based on morphological characters (Earlé *et al.* 1993; Campbell and Ellis, 2007; Peirce and Parsons, 2012; Vanstreels *et al.* 2016).

Assessing moisture of soil and material from nests

The glass bottles containing the fresh soil and nest material were weighed using an electronic scale (PS 4500/C/2, Radwag Wagi Elektroniczne, Radom, Poland) (combined wet and jar weight, for each of the samples). The weight of the empty jar was subtracted from the combined weight to obtain the wet weight of the soil and nest material. Thereafter, the sample was dried in an oven. Soil samples were dried at 105°C, while nest material was dried at 60°C, both for 24 hours (Gardner, 1965). The sample bottles were reweighed and thereafter the empty jar was subtracted to obtain the dry weight of the soil and nest material. The gravimetric (dry) nest material and soil water concentration was then calculated and expressed as the percentage of water in each of the samples (nest material and soil) in each nest (International Standards Organisation, 1993):

$$W_d = \frac{\text{weight of moist soil (g)} - \text{weight of dry soil (g)}}{\text{weight of dry soil (g)}} \text{x } 100$$

where W_d is the gravimetric (dry) soil water concentration

Statistical analysis

The effect of nest characteristics on nest microclimatic variables was assessed (mean soil temperature over a 30-day period from each nest; standard deviation of the soil temperature

records from each nest over a 30-day period, where higher values denote more variation of soil temperature; moisture of soil (%) and moisture of nest material (%)). The microclimatic variables were corrected for sampling period (SP1: autumn/winter 2016; SP2: spring 2016; and SP3: autumn/winter 2017) using generalised linear models (GLM) with a Gaussian error distribution using the glm() function in R. The effect of the different nest characteristics (nest type, occupancy, age, orientation, opening, distance from the coast, mean soil temperature, moisture of soil and moisture of nest material) corrected for sampling period on the abundance and prevalence of nest ectoparasites (ticks and fleas) was investigated. Parasite abundance data (number of individual parasites in nests regardless of whether or not the nest is infested; Bush *et al.* 1997) was modified by adding 1 value, log transformed and rounded, followed by testing for overdispersion (glm 'quasipoisson'). Zero-Inflated regression models (Negative Binomial for data overdispersion) were used to model data with excess zeros (Zeileis *et al.* 2008) using the zeroinfl function from the 'pscl' R package (Jackman, 2017). Parasite prevalence (number of nests infested by 1 or more individual parasites divided by the total number of nests examined; Bush *et al.* 1997) was modelled using glm 'binomial' (for presence/absence data). Regression models were ran with the reference at each nest type, however, we presented tables with the intercept in artificial nests for simplicity.

Once identified the nest characteristics that affected nest ectoparasite abundance and prevalence, association of in-nests ectoparasites (fleas and ticks together) with on-host ectoparasites (fleas and ticks) were explored using the spearman correlation test, after testing for data normality with the Shapiro test. Subsequently, the effect of nest type on penguin health parameters (body mass, chick body condition, haematocrit and total plasma protein) was assessed, correcting for the nest characteristics that were significant in the previous analysis, penguin age and on-host ectoparasites. GLM with a Gaussian error distribution was used for chick body condition and penguin total plasma protein, GLM Poisson distribution was used for haematocrit using the glm() function in R, and GLM Negative Binomial distribution was used for body mass using the function glm.nb(). The latter was calculated using the 'MASS' R package (Venables and Ripley, 2002). Although full models (all variables included) were used for hypothesis testing, a backward model selection test was performed using the Akaike's information criterion (AIC) and a chi-squared test to compare the full models with the best models from the AIC. Haemoparasite prevalence in adult

penguins and chicks between the different nest types were compared using proportion tests. All statistical tests and plot design were conducted in R 3·4·3 (R Core Development Team, 2017).

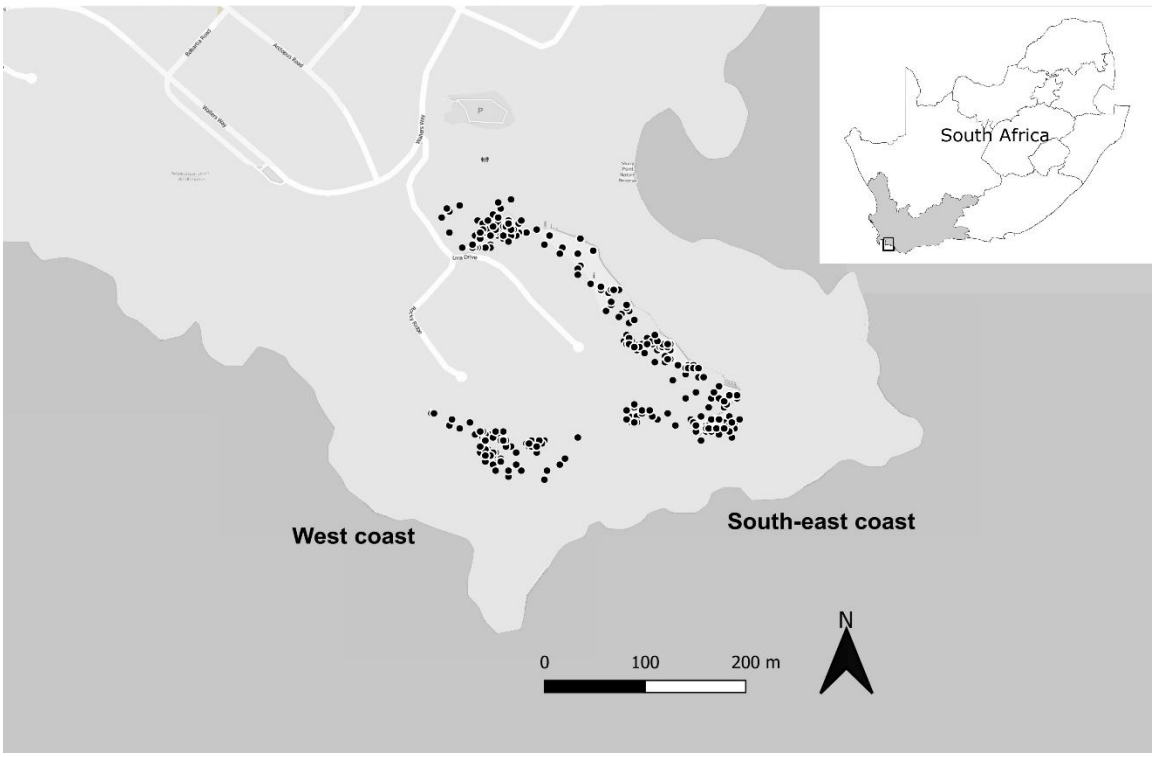

Figure 4.1. The Stony Point penguin colony in Betty's Bay, South Africa. Black dots are African penguin nests sampled during the three sample periods (i.e. autumn/winter 2016, spring 2016 and autumn/winter 2017).

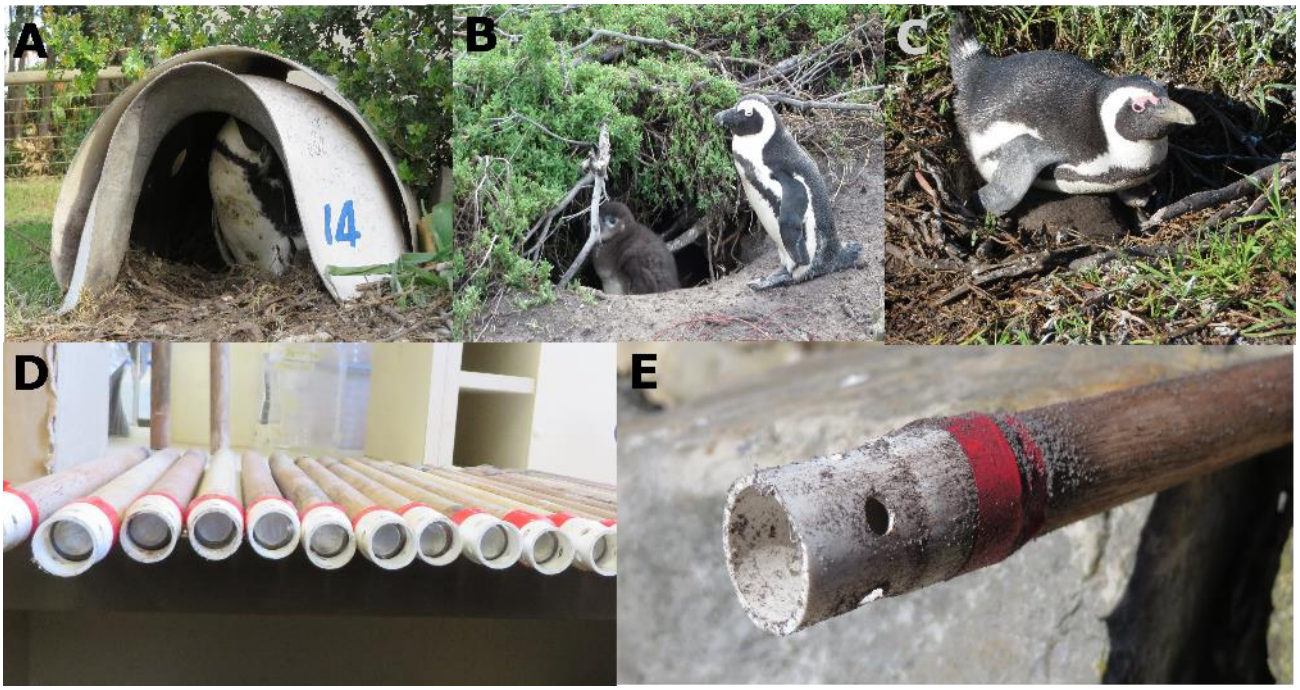

Figure 4.2. Nest types that were included in the study: artificial (A), natural covered (B) and natural open nests (C). Front (D) and side view (E) of plastic pipe used to insert the iButtons in the nest soil.

Table 4.1. Sample size per bird age (adult penguins and chicks) in each of the nest type across seasons in the Stony Point African penguin colony, South Africa.

Nest Type	Autumn/winter 2016	Spring 2016	Autumn/winter 2017	Total
Artificial nests				
Adults	10	1	9	20
Chicks	28	8	28	64
Natural covered				
Adults	5	4	9	18
Chicks	29	12	28	69
Natural open				
Adults	7	3	2	12
Chicks	26	4	29	59

Results

Fleas (*Parapsyllus humboldti*) and soft ticks (*Ornithodoros capensis* s. s.) were recorded in penguin nests and on penguins. Parasite infestations (mean abundance and prevalence) in nests were 13.36±1.68 and 64% for *P. humboldti* and 11.38±3.84 and 74% for *O. capensis* (s. s.). Parasite infestations (mean abundance and prevalence) on penguins were 5.20±0.34 and 81% for *P. humboldti* and 57±0.11 and 21% for *O. capensis* (s. s.). Haemoparasites were recorded in 56% of the penguins.

Microclimate and nest characteristics

The microclimatic conditions (mean temperature of nest soil, soil temperature SD, soil moisture and nest material moisture) associated with various nest characteristics are presented in Table 4.2. Further information on the mean values and proportions obtained from nest microclimatic conditions and characteristics recorded per nest type and sampling season are presented in Supplementary Table S4.1. None of the full models used in the analysis differed significantly from the best models estimated with the AIC (Supplementary Table S4.2). Mean soil temperature was significantly higher in artificial nests compared to natural covered (*t*-statistic $p<0.001$) and natural open (*t*-statistic $p<0.01$) nests. Soil temperature SD was significantly higher in artificial nests compared to natural covered (*t*-statistic $p<0.01$) and natural open (*t*-statistic $p<0.05$) nests. Soil moisture and nest material moisture in artificial nests were not significantly different from natural covered nests (*t*-statistic $p>0.05$ in both cases). However, artificial nests and natural covered nests were significantly drier compared to natural open nests (soil moisture *t*-statistic $p<0.001$ and $p<0.01$, respectively, and nest material moisture *t*-statistic $p<0.001$ and $p<0.01$, respectively). Mean soil temperature (*t*-statistic $p<0.01$) and nest material moisture (*t*-statistic $p<0.05$) were higher in active compared to non-active nests. Soil moisture was higher in older nests (*t*-statistic $p<0.05$) and in nests further away from the south-east coast line (*t*-statistic $p<0.01$). Mean soil temperature and temperature SD were significantly lower (*t*-statistic $p<0.05$ in both cases) in nests with wider entrances. When comparing between seasons and years, soil temperature was lower (*t*-statistic $p<0.001$) and nest material wetter (*t*-statistic $p<0.001$) in autumn 2016 compared to spring in 2016, while soil temperature (*t*-statistic $p<0.001$) and

nest material moisture (*t*-statistic $p<0.05$) were both lower in autumn 2016 compared to autumn 2017.

Nest characteristics and ectoparasite abundance and prevalence in nests

The effect of nest characteristics on the total nest ectoparasite (fleas and ticks) abundance and prevalence is presented in Table 4.3. The majority of the full models used in the analysis did not differ significantly from the best models estimated with the AIC (Supplementary Table S4.3). Artificial nests harboured a significantly higher abundance and prevalence of total ectoparasites (*z*-statistic $p<0.001$ and $p<0.01$, respectively) (Fig. 4.3A), fleas (*z*-statistic $p<0.001$ and $p<0.001$, respectively) and ticks (*z*-statistic $p<0.001$ and $p<0.01$, respectively) compared to natural open nests. Artificial nests also harboured a higher abundance of ticks (*z*-statistic $p<0.05$) compared to natural covered nests, while natural covered nests harboured a higher abundance of total ectoparasites (*z*-statistic $p<0.001$) and a higher abundance and prevalence of fleas (*z*-statistic $p<0.01$ and $p<0.001$, respectively) compared to natural open nests. Active nests harboured a higher abundance of fleas (*z*-statistic $p<0.001$) compared to non-active nests (Fig. 4.3B). The distance of the nests to the coast also affected parasite infestations in nests: total ectoparasite abundance increased with a decrease in distance from the south-east coast (*z*-statistic $p<0.05$) (Fig. 4.3C) and flea prevalence also increased (*z*-statistic $p<0.05$) with a decrease in distance from the west coast. Total nest ectoparasite abundance (Fig. 4.3D) and flea prevalence increased with mean soil temperature (both *z*-statistic $p<0.05$). In addition, total ectoparasite abundance (Fig. 4.3E) and flea abundance and prevalence decreased with an increase in nest soil moisture (all *z*-statistic $p<0.001$). A similar pattern was recorded for nest material: total ectoparasite abundance (*z*-statistic $p<0.01$) (Fig. 4.3F) and flea prevalence (*z*-statistic $p<0.05$) decreased with an increase in nest material moisture.

Nest type, penguin health and haemoparasite infestations

There was a significant positive correlation between ectoparasite infestation in nests and on penguins ($r_{Spearman}=0.31$, $p<0.01$) (Fig. 4.4). The effect of nest type on the clinical parameters of penguins is presented in Table 4.4. All the full models used in the analysis did not differ significantly from the best models estimated with the AIC (Supplementary Table S4.4).

Penguin body mass is the only clinical parameter that was affected by nest type, where penguins in natural open nests recorded a lower body mass compared to penguins in artificial (z-statistic $p<0.05$) and natural covered nests (z-statistic $p<0.05$). Penguin body mass and chick condition decreased with an increase in nest soil temperature (z-statistic $p<0.001$ and t-statistic $p<0.01$, respectively). Penguin body mass and total plasma protein increased with moisture of nest material (z-statistic $p<0.01$ and t-statistic $p<0.05$, respectively). In addition, penguin body mass and total plasma protein was lower in spring 2016 compared to autumn/winter 2016 (z-statistic $p<0.05$ and t-statistic $p<0.001$, respectively) and lower in autumn/winter 2017 compared to autumn/winter 2016 (z-statistic $p<0.001$ and t-statistic $p<0.001$, respectively).

The prevalence of haemoparasites (Piroplasmorida/Haemospororida and Spirochaetales combined) in adult penguins and chicks varied between the nest types, though the difference was not significant (Fig. 4.5). It is interesting to note that haemoparasites were recorded in adult penguins in artificial and natural covered nests while none were recorded in penguins in natural open nests. This pattern was however not evident for chicks.

Table 4.2. Relationship between nest characteristics and the nest microclimatic conditions of African penguins. Sampling seasons: autumn/winter 2016 (SP1); spring 2016 (SP2); and autumn/winter 2017 (SP3). Family of regression models according to data distribution: glm 'gaussian'. Significant values: ***=<0.001, **=0.001-0.01, *=0.01-0.05, ·=non-significant.

Y= Mean temperature (glm 'gaussian')					
Intercept	Independent variable	Estimate	Standard error	t value	p-value
Artificial nests	Natural covered nests	-0.8736402	0.2516725	-3.471	***
Artificial nests	Natural open nests	-0.7380691	0.2496863	-2.956	**
Non-active nest	Active nest	0.6696781	0.2169366	3.087	**
	Nest age	0.0824688	0.1523755	0.541	.
	Distance to south-east coast	0.0004752	0.0011869	0.4	.
	Distance to west coast	0.0010276	0.0011068	0.928	.
SP1	SP2	3.7878014	0.2559632	14.798	***
SP1	SP3	1.1084323	0.2495571	4.442	***
Nest position "leeward"	Nest position "windward"	-0.184637	0.266176	-0.694	.
	Nest opening	-0.018687	0.008592	-2.175	*
SP1	SP2	3.262136	0.327901	9.949	***
SP1	SP3	1.23494	0.316428	3.903	***
Y= Temperature SD (glm 'gaussian')					
Intercept	Independent variable	Estimate	Standard error	t value	p-value
Artificial nests	Natural covered nests	-0.4348113	0.1438705	-3.022	**
Artificial nests	Natural open nests	-0.2854484	0.1427351	-2	*
Non-active nest	Active nest	0.1986981	0.1240135	1.602	.
	Nest age	-0.0196898	0.0871066	-0.226	.
	Distance to south-east coast	0.0001495	0.0006785	0.22	.
	Distance to west coast	-0.000191	0.0006327	-0.302	.
SP1	SP2	-0.0935428	0.1463233	-0.639	.
SP1	SP3	-0.1166255	0.1426612	-0.817	.
Nest position "leeward"	Nest position "windward"	0.028599	0.156025	0.183	.
	Nest opening	-0.012565	0.005036	-2.495	*
SP1	SP2	-0.3167	0.192206	-1.648	.
SP1	SP3	-0.092614	0.18548	-0.499	.
Y= Soil moisture (glm 'gaussian')					
Intercept	Independent variable	Estimate	Standard error	t value	p-value
Artificial nests	Natural covered nests	6.78738	4.52023	1.502	.
Artificial nests	Natural open nests	27.32061	4.51935	6.045	***
Non-active nest	Active nest	-3.36109	3.94502	-0.852	.

	Nest age	6.14986	2.74674	2.239	*
	Distance to south-east coast	0.06443	0.02154	2.991	**
	Distance to west coast	-0.01432	0.01987	-0.721	.
SP1	SP2	-1.90715	4.55172	-0.419	.
SP1	SP3	-5.25079	4.42822	-1.186	.
Nest position "leeward"	Nest position "windward"	1.75414	3.9105	0.449	.
	Nest opening	-0.01679	0.12916	-0.13	.
SP1	SP2	-0.7586	4.7297	-0.16	.
SP1	SP3	-4.31788	4.55389	-0.948	.

Y= Nest material moisture (glm 'gaussian')

Intercept	Independent variable	Estimate	Standard error	t value	p-value
Artificial nests	Natural covered nests	4.00E+00	3.23E+00	1.238	.
Artificial nests	Natural open nests	1.22E+01	3.17E+00	3.835	***
Non-active nest	Active nest	7.21E+00	2.78E+00	2.594	*
	Nest age	1.60E-01	1.90E+00	0.084	.
	Distance to south-east coast	1.28E-02	1.54E-02	0.827	.
	Distance to west coast	6.22E-04	1.43E-02	0.044	.
SP1	SP2	-1.52E+01	3.25E+00	-4.688	***
SP1	SP3	7.88E+00	3.15E+00	2.499	*
Nest position "leeward"	Nest position "windward"	-1.402382	3.10233	-0.452	.
	Nest opening	-0.009414	0.100182	-0.094	.
SP1	SP2	-13.683996	3.808834	-3.593	***
SP1	SP3	8.697695	3.691539	2.356	*

Table 4.3. Relationship between nest characteristics and the abundance and prevalence of ectoparasites in African penguin nests (fleas and soft ticks combined and separately). Sampling seasons: autumn/winter 2016 (SP1); spring 2016 (SP2); and autumn/winter 2017 (SP3). Type and family of regression models according to data distribution: Zero Inflated Negative Binomial (ZINB) and glm 'binomial'. Significant values: ***=<0.001, **=0.001-0.01, *=0.01-0.05, ·=non-significant.

Y= Total nest ectoparasite abundance (ZINB)					
Intercept	Independent variable	Estimate	Standard error	z value	p-value
Artificial nests	Natural covered nests	-0.1290801	0.0683067	-1.89	.
Artificial nests	Natural open nests	-0.4356107	0.0707775	-6.155	***
Non-active nest	Active nest	0.1178958	0.0658615	1.79	.
	Nest age	0.0461955	0.0422915	1.092	.
	Distance to south-east coast	-0.0006678	0.0003383	-1.974	*
	Distance to west coast	-0.0002551	0.0003072	-0.831	.
SP1	SP2	0.3363768	0.0773748	4.347	***
SP1	SP3	0.5069372	0.0688692	7.361	***
Nest position "leeward"	Nest position "windward"	-0.070551	0.070491	-1.001	.
	Nest opening	-0.004319	0.002319	-1.862	.
SP1	SP2	0.221659	0.089981	2.463	*
SP1	SP3	0.435262	0.079432	5.48	***
	Temperature soil (mean)	0.055084	0.022515	2.447	*
	Temperature soil (SD)	-0.011761	0.041101	-0.286	.
	Moisture soil	-0.004605	0.001233	-3.735	***
	Moisture nest material	-0.005176	0.001934	-2.677	**
SP1	SP2	-0.056582	0.120979	-0.468	.
SP1	SP3	0.411245	0.090139	4.562	***
Y= Total nest ectoparasite prevalence (glm'binomial')					
Intercept	Independent variable	Estimate	Standard error	z value	p-value
Artificial nests	Natural covered nests	-1.0043797	0.5438927	-1.847	.
Artificial nests	Natural open nests	-1.624803	0.538577	-3.017	**
Non-active nest	Active nest	0.1772134	0.4730453	0.375	.
	Nest age	-0.3304501	0.2918102	-1.132	.
	Distance to south-east coast	0.0014334	0.0022639	0.633	.
	Distance to west coast	-0.0003028	0.0021846	-0.139	.
SP1	SP2	2.6325087	0.676953	3.889	***
SP1	SP3	2.4700835	0.5315754	4.647	***
Nest position "leeward"	Nest position "windward"	-0.34439	0.537924	-0.64	.
	Nest opening	0.001226	0.017812	0.069	.

SP1	SP2	2.945492	1.067177	2.76	**
SP1	SP3	2.488027	0.772626	3.22	**
	Temperature soil (mean)	0.3173851	0.2145118	1.48	.
	Temperature soil (SD)	0.0009744	0.4114758	0.002	.
	Moisture soil	-0.0110247	0.0085126	-1.295	.
	Moisture nest material	-0.0051313	0.0144976	-0.354	.
SP1	SP2	1.0723699	1.0287059	1.042	.
SP1	SP3	2.3193127	0.8258034	2.809	**

Y= Flea abundance (ZINB)

Intercept	Independent variable	Estimate	Standard error	z value	p-value
Artificial nests	Natural covered nests	-0.0704353	0.0884515	-0.796	.
Artificial nests	Natural open nests	-0.3472928	0.1009545	-3.44	***
Non-active nest	Active nest	0.3165093	0.0927832	3.411	***
	Nest age	0.0744642	0.0593849	1.254	.
	Distance to south-east coast	-0.0006978	0.0004935	-1.414	.
	Distance to west coast	-0.0001175	0.0004187	-0.281	.
SP1	SP2	0.0197593	0.1131871	0.175	.
SP1	SP3	0.4526582	0.0958505	4.723	***
Nest position "leeward"	Nest position "windward"	-0.0389786	0.0903154	-0.432	.
	Nest opening	0.0005669	0.0031979	0.177	.
SP1	SP2	-0.1214928	0.1224525	-0.992	.
SP1	SP3	0.3759363	0.1024153	3.671	***
	Temperature soil (mean)	0.025433	0.031898	0.797	.
	Temperature soil (SD)	0.001162	0.050737	0.023	.
	Moisture soil	-0.00921	0.002038	-4.519	***
	Moisture nest material	-0.002359	0.00265	-0.89	.
SP1	SP2	-0.318192	0.161995	-1.964	*
SP1	SP3	0.32666	0.125604	2.601	**

Y= Flea prevalence (glm'binomial')

Intercept	Independent variable	Estimate	Standard error	z value	p-value
Artificial nests	Natural covered nests	-0.601788	0.366481	-1.642	.
Artificial nests	Natural open nests	-1.886391	0.370716	-5.089	***
Non-active nest	Active nest	-0.092827	0.325335	-0.285	.
	Nest age	0.067258	0.207388	0.324	.
	Distance to south-east coast	0.001481	0.001581	0.936	.
	Distance to west coast	-0.003669	0.001587	-2.312	*
SP1	SP2	1.192062	0.355312	3.355	***

SP1	SP3	2.052953	0.345186	5.947	***
Nest position "leeward"	Nest position "windward"	-0.13657	0.36987	-0.369	.
	Nest opening	-0.01672	0.01142	-1.464	.
SP1	SP2	0.92941	0.41296	2.251	*
SP1	SP3	2.12606	0.46427	4.579	***
	Temperature soil (mean)	0.331809	0.137304	2.417	*
	Temperature soil (SD)	0.309159	0.292242	1.058	.
	Moisture soil	-0.02588	0.006922	-3.739	***
	Moisture nest material	-0.025942	0.010214	-2.54	*
SP1	SP2	-0.192691	0.661327	-0.291	.
SP1	SP3	1.987421	0.498801	3.984	***

Y= Tick abundance (ZINB)

Intercept	Independent variable	Estimate	Standard error	z value	p-value
Artificial nests	Natural covered nests	-0.2075204	0.0868179	-2.39	*
Artificial nests	Natural open nests	-0.3252452	0.0880674	-3.693	***
Non-active nest	Active nest	-0.1147275	0.0761002	-1.508	.
	Nest age	-0.0172925	0.0531945	-0.325	.
	Distance to south-east coast	-0.0007064	0.0004209	-1.678	.
	Distance to west coast	-0.0001941	0.0003767	-0.515	.
SP1	SP2	0.3957074	0.0927279	4.267	***
SP1	SP3	0.316488	0.0895344	3.535	***
Nest position "leeward"	Nest position "windward"	-0.0854	0.096979	-0.881	.
	Nest opening	-0.003975	0.002957	-1.344	.
SP1	SP2	0.386046	0.120383	3.207	**
SP1	SP3	0.302443	0.113323	2.669	**
	Temperature soil (mean)	0.04077	0.0290158	1.405	.
	Temperature soil (SD)	0.0163409	0.0570676	0.286	.
	Moisture soil	-0.0003483	0.0014888	-0.234	.
	Moisture nest material	-0.0046503	0.0025564	-1.819	.
SP1	SP2	0.1469346	0.1607348	0.914	.
SP1	SP3	0.3677228	0.1208544	3.043	**

Y= Tick prevalence (glm'binomial')

Intercept	Independent variable	Estimate	Standard error	z value	p-value
Artificial nests	Natural covered nests	-0.70471	0.377974	-1.864	.
Artificial nests	Natural open nests	-0.981023	0.373684	-2.625	**
Non-active nest	Active nest	-0.323537	0.375295	-0.862	.
	Nest age	-0.237157	0.215806	-1.099	.

	Distance to south-east coast	-0.002829	0.001691	-1.673	.
	Distance to west coast	0.001747	0.001602	1.091	.
SP1	SP2	2.778365	0.571581	4.861	***
SP1	SP3	1.079565	0.30932	3.49	***
Nest position "leeward"	Nest position "windward"	-0.7530201	0.3882047	-1.94	.
	Nest opening	0.0009948	0.01261	0.079	.
SP1	SP2	3.9337551	1.055637	3.726	***
SP1	SP3	1.0586004	0.3802095	2.784	**
	Temperature soil (mean)	-0.0116241	0.1339795	-0.087	.
	Temperature soil (SD)	-0.1288001	0.2468579	-0.522	.
	Moisture soil	0.0004723	0.0068042	0.069	.
	Moisture nest material	-0.0117809	0.01005	-1.172	.
SP1	SP2	2.5419114	0.8309387	3.059	**
SP1	SP3	1.1213923	0.4487969	2.499	*

Table 4.4. Relationship between nest characteristics and the clinical parameters of African penguins. Sampling seasons: autumn/winter 2016 (SP1); spring 2016 (SP2); and autumn/winter 2017 (SP3). Type and family of regression models according to data distribution: glm 'neg.bin', glm 'poisson' and glm 'gaussian'. Significant values: ***=<0.001, **= 0.001-0.01, *=0.01-0.05, ·=non-significant.

Y= Penguin body mass (glm 'neg.bin')					
Intercept	Independent variable	Estimate	Standard error	z value	p-value
Artificial nests	Natural covered nests	-0.0069408	0.0482334	-0.144	.
Artificial nests	Natural open nests	-0.1405466	0.0559531	-2.512	*
	Distance to south-east coast	0.0002936	0.0002224	1.32	.
	Distance to west coast	-0.0001713	0.0002267	-0.756	.
	Temperature soil (mean)	-0.0449632	0.0126083	-3.566	***
	Moisture soil	0.000115	0.0008306	0.138	.
	Moisture nest material	0.0026388	0.0010072	2.62	**
	Ectoparasites on penguins	-0.0080158	0.0038315	-2.092	*
SP1	SP2	-0.186106	0.0761394	-2.444	*
SP1	SP3	-0.1713386	0.0449688	-3.81	***
Adult	Chick	-0.295375	0.0487506	-6.059	***
Y= Chick body condition (glm 'gaussian')					
Intercept	Independent variable	Estimate	Standard error	t value	p-value
Artificial nests	Natural covered nests	0.0696965	0.0885661	0.787	.
Artificial nests	Natural open nests	-0.0578195	0.097913	-0.591	.
	Distance to south-east coast	0.0006733	0.0003856	1.746	.
	Distance to west coast	-0.0004465	0.0003949	-1.131	.
	Temperature soil (mean)	-0.0609134	0.0228494	-2.666	**
	Moisture soil	0.0002908	0.0014239	0.204	.
	Moisture nest material	0.0017002	0.0017581	0.967	.
	Ectoparasites on penguins	-0.0094037	0.0061742	-1.523	.
SP1	SP2	-0.1982155	0.1329082	-1.491	.
SP1	SP3	0.097287	0.0835226	1.165	.
Y= Penguin haematocrit (glm 'poisson')					
Intercept	Independent variable	Estimate	Standard error	z value	p-value
Artificial nests	Natural covered nests	0.04401	0.0385	1.143	.
Artificial nests	Natural open nests	-6.27E-02	0.04588	-1.367	.
	Distance to south-east coast	-5.28E-05	0.0001776	-0.298	.
	Distance to west coast	-1.88E-04	0.0001842	-1.023	.
	Temperature soil (mean)	-9.17E-03	0.01035	-0.886	.
	Moisture soil	-4.63E-04	0.0007167	-0.646	.

	Moisture nest material	8.17E-04	0.0008056	1.014	.
	Ectoparasites on penguins	-6.01E-03	0.003287	-1.827	.
SP1	SP2	6.53E-03	0.06263	0.104	.
SP1	SP3	-3.34E-02	0.03556	-0.938	.
Adult	Chick	-4.22E-01	0.03828	-11.035	***

Y= Penguin total plasma protein (glm 'gaussian')

Intercept	Independent variable	Estimate	Standard error	t value	p-value
Artificial nests	Natural covered nests	-0.005558	0.132	-0.042	.
Artificial nests	Natural open nests	-0.2118	0.1497	-1.415	.
	Distance to south-east coast	-0.0002548	0.0005918	-0.43	.
	Distance to west coast	4.283E-06	0.0006194	0.007	.
	Temperature soil (mean)	-0.03506	0.03445	-1.018	.
	Moisture soil	0.00382	0.002352	1.624	.
	Moisture nest material	0.005896	0.002684	2.197	*
	Ectoparasites on penguins	0.007762	0.01036	0.749	.
SP1	SP2	-1.954	0.2072	-9.432	***
SP1	SP3	-1.974	0.1211	-16.298	***
Adult	Chick	-1.554	0.1357	-11.452	***

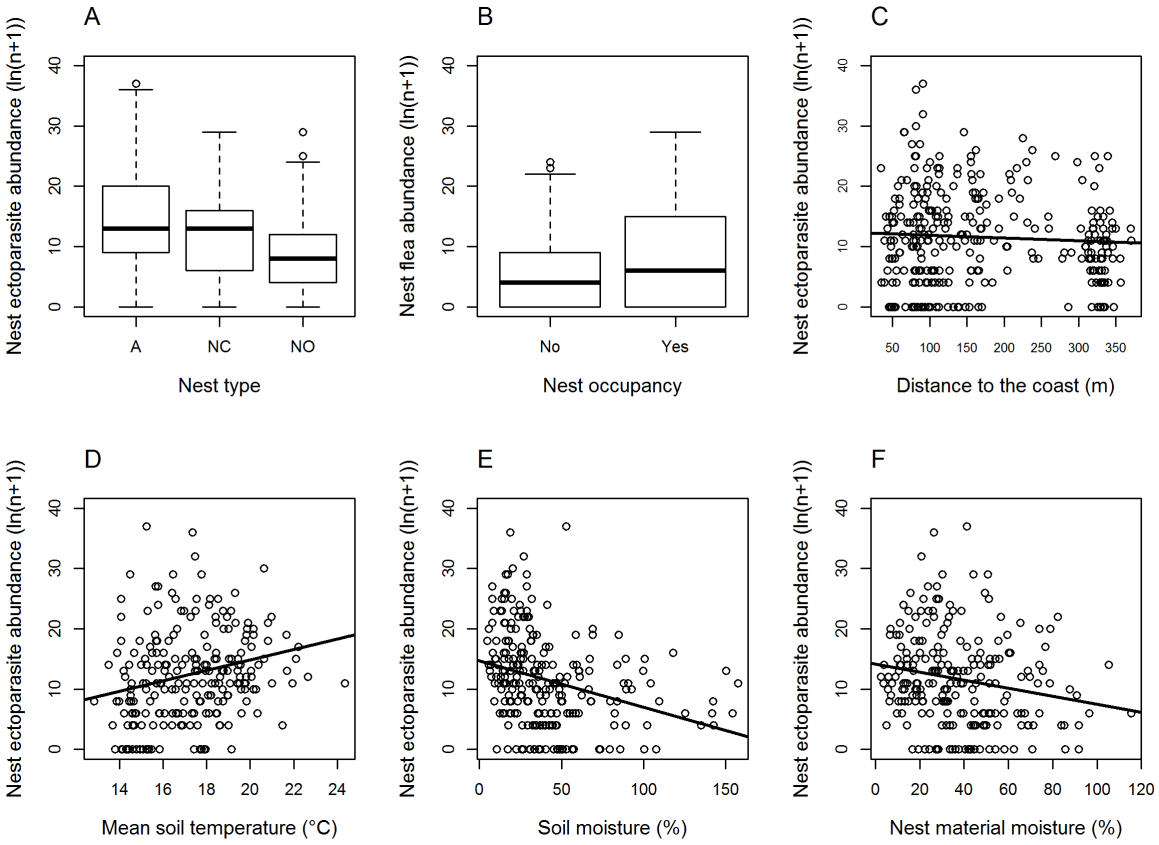

Figure 4.3. Relationship between nest characteristics and ectoparasite abundance in African penguin nests. Nest type: 'A' artificial, 'NC' natural covered and 'NO' natural open (A), nest occupancy: 'no' inactive nests and 'yes' active nests (B), distance to the south-east coast (C), mean soil temperature in nest (D), moisture of soil in nest (E), and moisture of nest material (F).

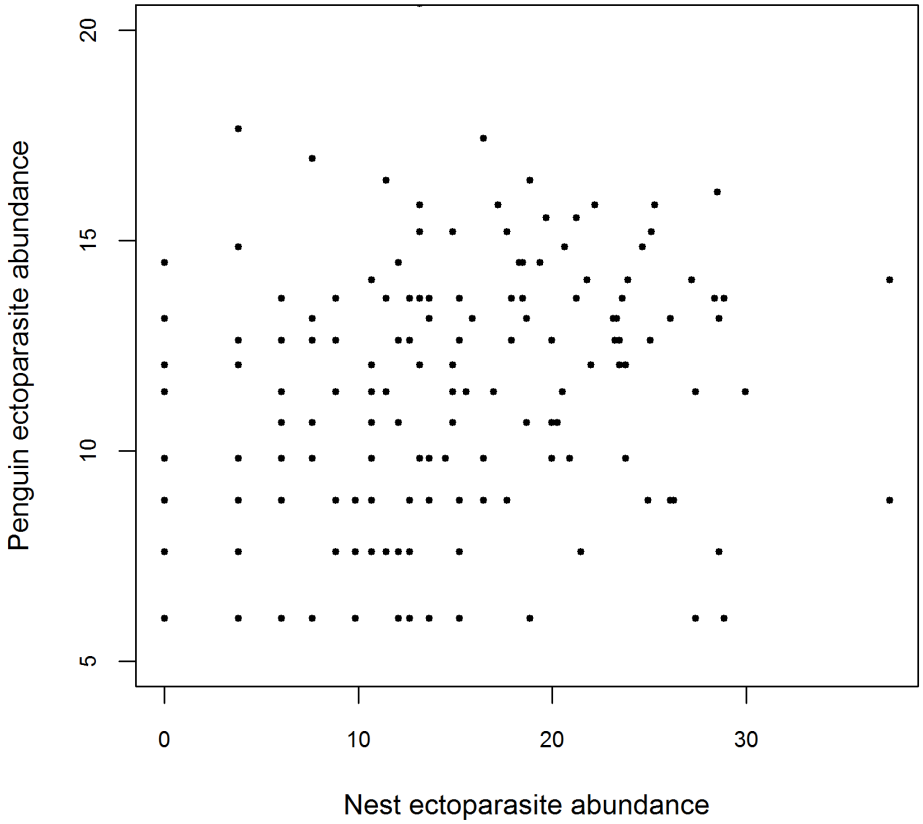

Figure 4.4. Spearman correlation ($r_{Spearman}$=0.31, p<0.01) between the abundance of ectoparasites in nest (fleas and soft ticks) and ectoparasites on adult and chick African penguins (fleas and soft ticks) at Stony Point colony during 2016 and 2017.

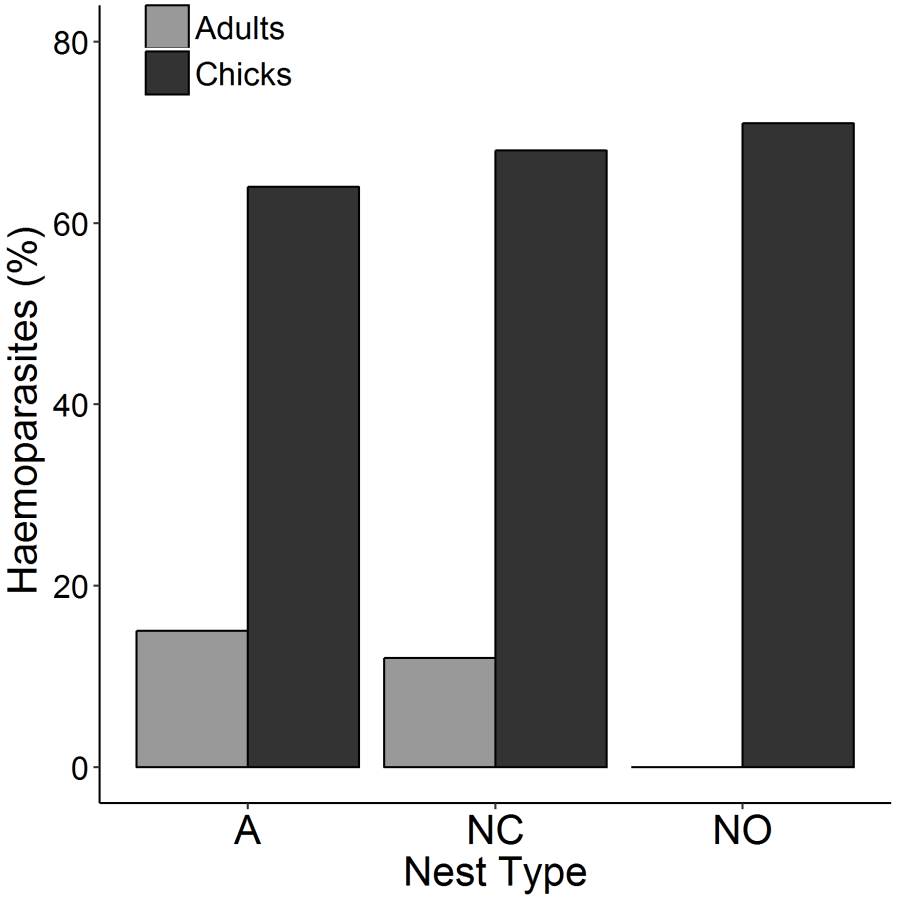

Figure 4.5. Prevalence (%) of haemoparasites (order Piroplasmids/Haemosporida and Spirochaetales) in adult African penguins and chicks per nest type (A: artificial, NC: natural covered, NO: natural open) at the Stony Point colony in 2016 and 2017. Sample sizes: A=20 adults and 63 chicks, NC=17 adults and 66 chicks and NO=11 adults and 58 chicks.

Discussion

The effect of nest characteristics and microclimate on nest ectoparasites

Nest type was the most consistent nest characteristic exerting an effect on the abundance and prevalence of ectoparasites in nests. In general, artificial nests harboured a higher abundance and prevalence of fleas and soft ticks than natural open nests. Artificial nests also harboured a higher abundance of soft ticks compared to natural covered nests. These patterns support previous studies that recorded a higher abundance of ectoparasites (in this case blowflies) in artificial nests of the eastern bluebird (*Sialia sialis*) compared to natural nests (Pinkowski, 1977). In addition, Wesołowski and Stańska (2001) recorded a higher prevalence of hen fleas

in nest boxes than in the natural holes of the marsh tit (*Parus palustris*) and flycatchers (*Ficedula* spp.).

The high ectoparasite abundance and prevalence in artificial compared to natural nests can be explained by the microclimatic conditions associated with the different nest types. The soil temperature in artificial nests were significantly higher (mean soil temperature and standard variation) compared to natural covered and natural open nests. The material used for nest construction affects the environment in nests, and fibreglass or cement-fibre nests are less porous, which increases the insularity by preventing heat loss in nests (Deeming and Mainwaring, 2015). This is in agreement with Lei *et al.* (2014) that found that the ambient temperature was consistently higher and humidity lower in African penguins nests made from cement and especially fiberglass compared to natural burrows. In addition, poor ventilation in artificial African penguin nests can also restrict the cooling effect of the ocean (Griffin, 2005). The abovementioned pattern is also supported by the fact that, irrespective of nest type, total ectoparasite (fleas and ticks combined) abundance and flea (adults and larvae) prevalence were significantly higher in nests with higher soil temperatures in the present study. Fleas and ticks clearly benefit from higher nest temperatures. In particular, fleas can increase their frequency of blood intake and digestion of blood meals when ambient temperature increases (Krasnov, 2008), while a rise in temperature can trigger the emergence of imagos from cocoons (Humphries, 1968; Marshall, 1981). Furthermore, higher temperatures can facilitate the survival of flea larvae such as the larvae of the stick tight flea (*Echidnophaga gallinacea*) which dies when the temperature is below 10°C (Marshall, 1981), while an increase in temperature stimulates mating of some flea species (e.g. northern rat flea *Nosopsyllus fasciatus*) (Iqbal and Humphries, 1970). All of the above suggests that nest temperatures can induce fleas to complete their life cycle in a shorter period of time (Marshall, 1981). It seems that soft ticks have a similar response to higher nest temperatures, and in particular pre-oviposition and oviposition periods shorten with an increase in temperature (Diehl *et al.* 1982). Soft ticks are also morphologically adapted to resist higher temperatures since components of their epicuticle (cuticulin, polyphenol, wax and cement layers) provide an effective barrier against water loss (Lees, 1947).

The non-porous nature of the artificial nests also resulted in lower soil and nest material moisture compared to natural open nests. Open surface nests are more exposed to rain and

ocean spray, which would explain the higher moisture content. This is in agreement with previous studies on penguin colonies (e.g. royal penguins *Eudyptes schlegeli* and African penguins), which ascribed the wetter conditions in open nests to their exposure to severe weather conditions (Murray and Vestjens, 1967; Seddon and van Heezik, 1991). Hebda and Wesołowski (2012) also found that natural tree cavities are more humid and get soaked by rain, while artificial nest-boxes are drier. Studies on penguin (rockhopper penguins *Eudyptes chrysocome* and royal penguins) parasites indicated that *Parapsyllus magellanicus* fleas and *Ixodes uriae* ticks require a dry habitat to breed and often avoid wet or flooded places (Murray and Vestjens, 1967). In the present study, soil and nest material moisture had a similar effect on the total ectoparasite abundance and flea abundance and prevalence, irrespective of nest type. It has been recognized that the environmental water content (in the form of relative humidity or moisture) is a crucial factor for the development of fleas (Rothschild and Clay, 1952; Marshall, 1981). To avoid mortality, larvae and cocoons are able to absorb moisture from the environment, which is important for the development of adult fleas (Marshall, 1981). However, not all flea species benefit from high humidity. For example, the abundance of adult and larval stages of the hen flea (*Ceratophyllus gallinae*) infesting nests of the great tit (*Parus major*) were negatively affected by nest moisture (Eeva *et al.* 1994) and hen fleas more often colonised nests with lower humidity levels (Heeb *et al.* 2000). These findings, which are consistent with the negative relationship between nest moisture and flea abundance and prevalence demonstrated in the present study, might indicate the existence of a moisture threshold above which flea populations start to decrease.

Although there was no direct relationship between nest moisture and soft ticks, nest moisture did affect ticks when included in the total ectoparasite abundance. This supports a previous study that recorded an adverse effect of nest moisture on the abundance of *O. capensis* ticks in African penguin nests (Daturi, 1986). The authors argued that wet conditions in the nest might limit the tick movement and therefore tick population growth. Ticks also have the ability to absorb water vapour from the atmosphere (Knülle and Rudolph, 1982) and exhibit a range of critical humidity equilibria where they maintain a balance in their water level (Knülle and Wharton, 1964). For *O. capensis* the critical humidity equilibrium is ≤80% RH (Balashov and Filippova, 1964). However, when there is an excess of water in the environment unfed ticks can accumulate a higher amount of body water (Sauer

and Hair, 1971; Knülle and Rudolph, 1982). Water-saturated ticks respond positively to light stimulus and negatively to moisture, and this guides the tick out of the damp nest and possibly to the surrounding area (Knülle and Rudolph, 1982), which could also explain the lower abundance and prevalence of soft ticks in natural open nests and nests with a high moisture content in general. This pattern is also supported by the absence of tick-borne pathogens such as haemoparasites in adult penguins from natural open nests, while a prevalence of 15% and 12% were recorded in adult penguins from the drier artificial and natural covered nests, respectively.

In this study, ectoparasite loads also differed between the two types of natural nests with higher total ectoparasite and flea abundance and higher flea prevalence in natural covered compared to natural open nests. This pattern is also supported by a study on rockhopper penguins (*Eudyptes chrysocome*) where higher flea (*Parapsyllus magellanicus heardi*) infestations were recorded on penguins and in nests located in sheltered natural places (e.g. caves and under rocks) compared to nests in open areas (Murray and Vestjens, 1967). Soft ticks also seem to display this pattern in African penguin nests with higher soft tick abundance and prevalence in holes and burrows compared to open nests (Daturi, 1986). Natural covered nests use the natural soil of the colony and are covered by vegetation, creating a sheltered and shaded environment which is microclimatically more similar to burrows (Deeming and Mainwaring, 2015; López-Rull and Macías-Garcia, 2015). Comparative studies on the nest types used by African penguin recorded less variation of temperatures and humidity in natural burrows compared to open surface nests (Frost *et al.* 1976; Griffin, 2005; Lei *et al.* 2014). Natural open nests are more exposed to climatic conditions (such as sunlight, rain and wind) and extreme fluctuations in temperature (high increase during the day and decrease at night) can occur (Griffin, 2005; Sherley *et al.* 2012). In the present study, soil moisture was higher in natural open compared to natural cover nests, which is consistent with the more exposed conditions of open nests that was previously found.

The effect of nest distance from the coast on ectoparasites

The proximity of nests in relation to the coast line seemed to affect parasite infestations. In particular, total ectoparasite abundance and flea prevalence were higher in nests located

closer to the south-east coast (coast with prevalent winds during spring) and west coast (prevalent winds during winter), respectively. The examination of nest microclimatic conditions indicated that nests located closer to the south-east coast exhibited lower soil moisture (i.e. were drier) compared to nests that were further away. Nests located closer to the coast might be more ventilated, possibly due to prevailing coastal winds, which can facilitate heat loss of the nests (Deeming and Mainwaring, 2015). In Stony Point, nests located further inland were also more protected by the tree and shrub canopy (predominately *Acacia cyclops*, *Metrosideros excelsa* and *Searsia pyroides*) which would reduce wind speed and air circulation (Griffin, 2005). As discussed earlier, flea and tick infestations are negatively affected by high moisture content (Balashov and Filippova, 1964; Heeb *et al.* 2000). This would explain the pattern observed in the present study. However, there is another factor that may contribute to the spatial pattern observed in the Stony Point colony. A large population of Cape cormorant (*Phalacrocorax capensis*) nests on the south-east side of the colony every year (from September to March the following year; CapeNature unpubl. data), and is in close proximity to the penguin nests. Since the fleas and ticks recorded in this study are ectoparasites of seabirds (Jordan, 1942; Hoogstraal *et al.* 1985), it is possible that interspecies transmission of these parasites can occur in this part of the colony (Jordan, 1942; Hoogstraal *et al.*1976). In addition, the presence of nesting cormorants increases the density of seabirds in the area, thus offering more resources (food and nests) and facilitating ectoparasite transmission (Rothschild and Clay, 1952; Duffy, 1988).

The effect of nest occupancy on ectoparasites

Flea abundance was positively affected by the presence of a bird (adult or chick) in the nest (i.e. active nests). This result is consistent with the ecology of fleas. Adult fleas are the only parasitic stage in the life cycle and thus require the presence of a host for a blood meal and egg production (Bitam *et al.* 2010). Furthermore, flea larvae indirectly benefit from the presence of a host in the nest as their diet include host organic refuse (such as dung) and the larvae of some flea species consume the faeces of adult fleas (Rothschild and Clay, 1952; Rothschild, 1975). For example, du Feu (1987) observed that the emergence of fleas (*Dasypsyllus gallinulae* and *Ceratophyllus gallinae*) in nest boxes of blue tit (*Parus caeruleus*) and great tit coincided with the bird nesting activity. The presence of a host in the

nest also creates ideal microclimatic conditions as the physical presence of an endothermic host increases the nest temperature through heat radiation from the host's body (Rothschild and Clay, 1952). Fleas are attracted to body heat, which not only represents a source of food but also seems to stimulate oviposition and accelerates flea development (Kyrakova, 1960; López-Rull and Macías-Garcia, 2015). Indeed, the results of our study showed that the mean soil temperature in nests was higher in active nests compared to non-active nests. Tripet and Richner (1999) also recorded temperature of nests increased with the presence of blue tit in their nests. In their study, the temperature of nest material was 12.1°C and 17.4°C higher compared to outside temperatures during the incubation and nestling periods, respectively. Likewise, Humphries (1968) witnessed that the flea imago's (*Ceratophyllus* spp.) quickly emerged from their cocoons after a bird was in the nests providing heat and tactile stimuli, unlike deserted nests, in which the imago's remain in the cocoons for longer periods of time. The presence of a host also contributes to increase moisture levels in the nest (Heeb *et al.* 2000). This is supported by the present study, where a positive effect of active nests on nest material moisture was recorded. The humidity radiated from the penguin's body and its faeces (guano) creates a moist environment in the nest (Frost *et al.* 1976; Marshall, 1981). As discussed before, humidity levels are important for flea development (Rothschild and Clay, 1952) and the microclimatic conditions in active nests are more favourable for the development and survival of fleas.

Nest occupancy did not have a significant effect on the abundance and prevalence of ticks. These results are consistent with the life history of soft ticks and agree with Daturi (1986), who also found no relationship between *O. capensis* abundance and nest occupancy in African penguin nests. Soft ticks may be less dependent on the host than fleas, since they are highly resistant to the absence of a food source (Sonenshine, 1993). Soft ticks can fast for years (up to 18 years under experimental conditions) and still remain in the nest (Uspensky, 2008). Moreover, soft ticks are often found in sheltered surroundings of the nests as long as the microclimatic conditions are suitable for tick survival (Sonenshine, 1993). It is therefore expected that in the short term, the presence or absence of hosts in the nests will not significantly affect soft tick demography.

Effect of nest type on penguin health

The only clinical parameter affected by nest type was penguin body mass. Body mass represents the total weight of the penguin, which includes body fat and water content (Lima, 1986; McLean *et al.* 2018). Since artificial nests harboured a higher ectoparasite abundance and prevalence, we expected the health status of penguins in artificial nests to be more affected than those in natural nests. Surprisingly, penguins (adults and chicks) in natural open nests exhibited a lower body mass compared to penguins in artificial and natural covered nests. Although natural open nests harboured lower ectoparasite abundance and prevalence than artificial and natural covered nests, it is possible that the lower body mass is related to the fact that penguins in open nests are more exposed to climatic conditions such as heat, sunlight, wind and rainfall (Griffin, 2005; Kemper *et al.* 2007). For example, penguins in open nests are more susceptible to extreme variations in temperature and/or strong winds leading to heat stress or heat loss (Frost *et al.* 1976; Pichegru, 2013). In addition, in seasons of heavy rainfall, open nests are more exposed to flooding (Kemper *et al.* 2007). These factors can exert thermoregulation demands inducing variation in the metabolic rate to achieve either insulation (heat production) or conductance (heat loss) (Drent and Stonehouse, 1971; Lei *et al.* 2014). Penguins then need to use more energy resources to compensate for this metabolic demand, which may be reflected in the loss of body mass (Catry *et al.* 2011).

Natural open nests are also more exposed to predators (such as kelp gulls (*Larus dominicanus*) feeding on eggs and chicks; Cooper, 1974), and to human disturbance (a potential consequence of tourism; Lewis *et al.* 2012). These stress factors induce secretion of corticosterone in plasma, which has been found to be negatively associated with body mass due to the mobilisation of energy reserves (Harvey *et al.* 1974; Schoech *et al.* 1997; DuRant *et al.* 2010; Fokidis *et al.* 2011). Additionally, the extreme climatic conditions and disturbances that adult penguins experience in natural open nests can induce them to abandon their nests and chicks (Frost *et al.* 1976; Duffy, 1983; Griffin, 2005). African penguin chicks are dependent on their parent's presence in the nest to reach a stable body temperature and achieve thermoregulatory capacity after they reach ca. 400g of body weight (Erasmus and Smith, 1974). The desertion of nests by adults leaves chicks even more exposed to unfavourable weather conditions and lack of food resources, thus promoting weight loss and a deterioration of their general health status (Ricklefs, 1987; Mainwaring *et al.* 2014).

Animals that are in poor condition often have a weaker immune response against vector-borne diseases and pathogens (Møller *et al.* 1998; Hoi-Leitner *et al.* 2001). This may explain the slightly higher, though not significant, incidence of tick-transmitted haemoparasites in penguin chicks in natural open nest.

Although artificial nests per se seemed to have no adverse effect on health parameters of penguins, it is interesting to note that penguin body mass and chick condition appear to be adversely affected by high nest soil temperature, a factor more associated with artificial and natural covered than with natural open nests. Body mass and condition are indicative of the food received by the bird and its energy reserves (Brown and Sherry, 2006; Chastel *et al.* 1995; Lubbe *et al.* 2014). As explained above, an increase in temperature in the nest might involve the acceleration of metabolic rate, with the resultant depletion of energy reserves reflected in a decrease in body mass and condition (Catry *et al.* 2011). This has been experimentally tested in nests of chestnut-collared longspurs (*Calcarius ornatus*), where the nests facing towards the sun, and therefore receiving more heat during the day, yielded smaller chicks than nests less exposed to the sun and recording cooler temperatures (Lloyd and Martin, 2004). The importance of nest soil temperature is further highlighted in the seasonal response (autumn/winter 2016 vs. spring 2016), where spring (higher temperatures than autumn/winter) had a negative effect on penguin body mass. Together with the direct effect of high temperatures on penguin body mass (McLean *et al.* 2018), the spring season (October-November) also coincides with the start of the moulting period of adult African penguins (adults leave the colony for ca. 4 weeks) (Crawford *et al.* 2006). This represents an additional stress factor for chicks because during this season they receive less food and protection from their parents, which challenges their immune response and susceptibility to parasites (Hoi-Leitner *et al.* 2001).

The fact that artificial nests have a higher abundance and prevalence of ectoparasites might suggest a fundamental problem with artificial nests, which are currently used to create a suitable environment for birds to breed and counteract the current reduction of their natural habitats (Bolton *et al.* 2004; Sherley *et al.* 2012). Artificial nests have assisted conservation efforts of threatened seabird species in the past. For example, the breeding success of Madeiran storm petrel (*Oceanodroma castro*) has increased three times over three successive seasons when using artificial nest boxes (Bolton *et al.* 2004). Artificial burrows also provided

a continuous and stable breeding environment for yelkouan (*Puffinus yelkouan*) and Scopoli's (*Calonectris diomedea*) shearwaters, improving their reproductive success (Bourgeois *et al.* 2015). In African penguins, the use of artificial nests has assisted the improvement of habitats (more protected from predators and climatic events) to increase the reproductive success of the species. For example, artificial burrows made from plastic bins have increased African penguin chick survival compared to nests on the surface, under bushes or inside old buildings at breeding colonies in Namibia (Kemper *et al.* 2007). In South African colonies, the use of artificial nests has also increased nestling survival in African penguins (Sherley *et al.* 2012; Pichegru, 2013). However, it has also been observed that after deployment the frequency of use decreases over time potentially highlighting the colonisation of the nests by parasites (Loye and Carroll, 1998; Kemper *et al.* 2007). It is possible that parasites at Stony Point have not yet reached such level to show a clinical effect on penguins. However, once parasites abundance increase, the health of penguins in artificial and natural cover nests could be more compromised than those in natural open nests.

Conclusions

The study highlights the importance of nest characteristics in shaping the microclimatic conditions within penguin nests. In particular, artificial and natural covered nests are drier and warmer compared to natural open nests. Ectotherms such as soft ticks and fleas are sensitive to temperature and humidity, which clearly influences their abundance and prevalence in certain nest types. Although there is as yet no evidence that penguins in artificial nests are adversely affected by higher parasite infestations, it does appear that in general health parameters are influenced by microclimatic conditions that are more characteristic of artificial compared to natural open nests. Management strategies such as the use of artificial nests are generally implemented to facilitate breeding success of threatened and endangered bird species. Given that colony dynamics can vary within and between bird species it is advisable that the impact of these interventions on the ecosystem is assessed on a regular basis.

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Supplementary material

Table S4.1. Nest characteristics assessed for African penguins at the Stony Point penguin colony along the west coast of South Africa. The mean value (±SE) of and proportion (%) per nest type and sampling season is presented. Sampling seasons: autumn/winter 2016 (SP1); spring 2016 (SP2); and autumn/winter 2017 (SP3).

	Nest type			**Sampling season**		
Nest Characteristics	**Artificial**	**Natural covered**	**Natural open**	**SP1**	**SP2**	**SP3**
Microclimate (Mean±SE)						
Soil mean temperature (°C)	17.78 (±0.24)	16.81 (±0.22)	16.96 (±0.27)	15.51 (±0.19)	19.13 (±0.16)	16.65 (±0.18)
Soil temperature SD	1.91 (±0.11)	1.47 (±0.09)	1.62 (±0.08)	1.76 (±0.11)	1.62 (±0.09)	1.65 (±0.09)
Soil moisture (Wd)*	31.44 (±2.42)	34.7 (±2.76)	55.94 (±4.02)	41.05 (±3.16)	42.19 (±3.67)	38.51 (±3.19)
Nest material moisture (Wd)*	32.26 (±2.38)	35.99 (±2.30)	43.85 (±2.80)	40.73 (±2.11)	23.13 (±1.84)	48.52 (±2.71)
Occupancy (%)						
Active	74.51	74.76	70.87	82.57	46.91	83.05
Inactive	25.49	25.24	29.13	17.43	53.09	16.95
Age (%)						
1 (<1 year)	7.84	16.50	16.50	17.43	9.88	12.71
2 (1-3 years)	12.75	43.69	37.86	36.70	30.86	27.12
3 (>3 years)	79.41	39.81	45.63	45.87	59.26	60.17
Orientation (%)						
Windward	31.37	43.69	NA	25	59.26	34.67
Leeward	68.63	56.31	NA	75	40.74	65.33
Opening (cm) (Mean±SE)	29.88 (±0.37)	48.75 (±1.43)	NA	41.27 (±1.58)	38.41 (±1.99)	38.03 (±1.65)
Distance to the south-east coast (m) (Mean ±SE)	169.93 (±10.64)	169.08 (±10.06)	165.87 (±10.19)	157.17 (±10.09)	169.05 (±11.27)	178.03 (±9.61)
Distance to the west coast (m) (Mean±SE)	344.41 (±11)	353.54 (±10.21)	332.18 (±10.97)	346.57 (±9.76)	341.75 (±11.86)	341.54 (±10.74)

* Wd: gravimetric dry soil

Table S4.2. Model selection based on Akaike Information Criterion (AIC). Numbers in brackets represent the different scenarios derived from the full model (assessed in the study) to reach the best model (in bold). Significant differences between the full and the best model (p-value) according to Chi-square test. Independent variables: nest type (artificial, natural covered and natural open nests), nest occupancy (active or inactive), nest age (nests established within a year, nests established more than one and less than three years ago, and nests established more than three years ago), distance to the south-east coast (m), distance to the west coast (m), nest position (windward or leeward) nest opening (cm) and SP (sampling seasons: autumn/winter 2016, spring 2016 and autumn/winter 2017).

Model	AIC	Chisq	df	p-value
y= Mean temperature				
(1) temperature mean ~ nest type + nest occupancy + nest age + distance south-east + distance west + SP	846.8			
(2) temperature mean ~ nest type + nest occupancy + nest age + distance west + SP	844.97			
(3) temperature mean ~ nest type + nest occupancy + distance west + SP	843.19	3.61	2	>0.05
(1) temperature mean ~ nest position + nest opening + SP	580.89			
(2) temperature mean ~ nest opening + SP	579.39	1.5	1	>0.05
y= Nest material moisture				
(1) temperature SD ~ nest type + nest occupancy + nest age + distance south-east + distance west + SP	589.56			
(2) temperature SD ~ nest type + nest occupancy + nest age + distance south-east + distance west	586.31			
(3) temperature SD ~ nest type + nest occupancy + nest age + distance west	584.33			
(4) temperature SD ~ nest type + nest occupancy + nest age	582.38			
(5) temperature SD ~ nest type + nest occupancy	580.56	9	4	>0.05
(1) temperature SD ~ nest position + nest opening + SP	416.37			
(2) temperature SD ~ nest opening + SP	414.41			
(3) temperature SD ~ nest opening	413.5	2.87	2	>0.05

y= Soil moisture				
(1) moisture soil ~ nest type + nest occupancy + nest age + distance south-east + distance west + SP	2345.26			
(2) moisture soil ~ nest type + nest occupancy + nest age + distance south-east + distance west	2342.75			
(3) moisture soil ~ nest type + nest occupancy + nest age + distance south-east	2341.22			
(4) moisture soil ~ nest type + nest age + distance south-east	2340.12	5.14	3	>0.05
(1) moisture soil ~ nest position + nest opening + SP	1511.24			
(2) moisture soil ~ nest position + nest opening	1508.31			
(3) moisture soil ~ Nest position	1506.31			
(4) moisture soil ~ 1	1504.57	6.67	3	>0.05
y= Nest material moisture				
(1) moisture nest mat~ nest type + nest occupancy + nest age + distance south-east + distance west + SP	1950.75			
(2) moisture nest mat~ nest type + nest occupancy + nest age + distance south-east + SP	1948.75			
(3) moisture nest mat~ nest type + nest occupancy + distance south-east + SP	1946.76			
(4) moisture nest mat~ nest type + nest occupancy + SP	1945.93	4.82	3	>0.05
(1) moisture nest mat~ nest position + nest opening + SP	1255.28			
(2) moisture nest mat~ nest position + SP	1253.29			
(3) moisture nest mat~ SP	1251.51	3.77	2	>0.05

Table S4.3. Model selection based on Akaike Information Criterion (AIC). Numbers in brackets represent the different scenarios derived from the full model (assessed in the study) to reach the best model (in bold). Significant differences between the full and the best model (p-value) according to Chi-square test. Independent variables: nest type (artificial, natural covered and natural open nests), nest occupancy (active or inactive), nest age (nests established within a year, nests established more than one and less than three years ago, and nests established more than three years ago), distance to the south-east coast (m), distance to the west coast (m), nest position (windward or leeward) nest opening (cm), temperature mean (°C) + temperature SD (°C) + moisture of nest soil (%) + moisture of nest material (%) and SP (sampling seasons: autumn/winter 2016, spring 2016 and autumn/winter 2017).

Model	AIC	Chisq	df	p-value
y = Total nest ectoparasite abundance				
(1) total nest ectoparasite abundance ~ nest type + nest occupancy + nest age + distance south-east + distance west + SP	1883.2			
(2) total nest ectoparasite abundance ~ nest type + nest occupancy + nest age + distance south-east + SP	1879.93			
(3) total nest ectoparasite abundance ~ nest type + nest occupancy + distance south-east + SP	1878.2			
(4) total nest ectoparasite abundance ~ nest type + distance south-east + SP	1877.22	5.98	3	>0.05
(1) total nest ectoparasite abundance ~ nest position + nest opening + SP	1304.43			
(2) total nest ectoparasite abundance ~ nest opening + SP	1301.83			
(3) total nest ectoparasite abundance ~ SP	1301.58	2.85	2	>0.05
(1) total nest ectoparasite abundance ~ temperature mean + temperature SD + moisture soil + moisture nest mat + SP	1294.62			
(2) total nest ectoparasite abundance ~ temperature mean + moisture soil + moisture nest mat + SP	1290.7	3.92	1	>0.05
y = Total nest ectoparasite prevalence				
(1) total nest ectoparasite prevalence ~ nest type + nest occupancy + nest age + distance south-east + distance west + SP	205.74			
(2) total nest ectoparasite prevalence ~ nest type + nest occupancy + nest age + distance south-east + SP	203.76			
(3) total nest ectoparasite prevalence ~ nest type + nest age + distance south-east + SP	201.91			
(4) total nest ectoparasite prevalence ~ nest type + nest age + SP	200.34			
(5) total nest ectoparasite prevalence ~ nest type + SP	200.22	5.52	4	>0.05

(1) total nest ectoparasite prevalence ~ nest position+ nest opening + SP	120.66			
(2) total nest ectoparasite prevalence ~ nest position+ SP	118.67			
(3) total nest ectoparasite prevalence ~ SP	117.06	3.6	2	>0.05
(1) total nest ectoparasite prevalence ~ temperature mean + temperature SD + moisture soil + moisture nest mat + SP	117.41			
(2) total nest ectoparasite prevalence ~ temperature mean + moisture soil + moisture nest mat + SP	115.41			
(3) total nest ectoparasite prevalence ~ temperature mean + moisture soil + SP	113.53	3.88	2	>0.05
y = Flea abundance				
(1) flea abundance ~ nest type + nest occupancy + nest age + distance south-east + distance west + SP	1586.43			
(2) flea abundance ~ nest type + nest occupancy + distance south-east + distance west + SP	1584.09	2.34	1	>0.05
(1) flea abundance ~ nest position+ nest opening + SP	1180.49			
(2) flea abundance ~ nest opening + SP	1176.81			
(3) flea abundance ~ SP	1175.07	5.42	2	>0.05
(1) flea abundance ~ temperature mean + temperature SD + moisture soil + moisture nest mat + SP	1058.72			
(2) flea abundance ~ temperature mean + moisture soil + moisture nest mat + SP	1055.9	2.82	1	>0.05
y = Flea prevalence				
(1) flea prevalence ~ nest type + nest occupancy + nest age + distance south-east + distance west + SP	346.22			
(2) flea prevalence ~ nest type + nest age + distance south-east + distance west + SP	344.3			
(3) flea prevalence ~ nest type + distance south-east + distance west + SP	342.41			
(4) flea prevalence ~ nest type + distance west + SP	341.18	5.04	3	>0.05
(1) flea prevalence ~ nest position+ nest opening + SP	219.62			
(2) flea prevalence ~ nest opening + SP	217.75	1.87	1	>0.05
(1) flea prevalence ~ temperature mean + temperature SD + moisture soil + moisture nest mat + SP	211.47			
(2) flea prevalence ~ temperature mean + moisture soil + moisture nest mat + SP	210.69	0.78	1	>0.05
y = Tick abundance				
(1) tick abundance ~ nest type + nest occupancy + nest age + distance south-east + distance west + SP	1624.12			

(2) tick abundance ~ nest type + nest occupancy + distance south-east + distance west + SP	1621.44			
(3) tick abundance ~ nest type + nest occupancy + distance south-east + SP	1618.57			
(4) tick abundance ~ nest type + distance south-east + SP	1617.85	6.27	3	>0.05
(1) tick abundance ~ nest position+ nest opening + SP	1128.2			
(2) tick abundance ~ nest position+ SP	1125.99	2.21	1	>0.05
(1) tick abundance ~ temperature mean + temperature SD + moisture soil + moisture nest mat + SP	1175.44			
(2) tick abundance ~ temperature mean + temperature SD + moisture nest mat + SP	1171.5			
(3) tick abundance ~ temperature mean + moisture nest mat + SP	1167.85			
(4) tick abundance ~ moisture nest mat + SP	1167.32	8.12	3	<0.05
y = Tick prevalence				
(1) tick prevalence ~ nest type + nest occupancy + nest age + distance south-east + distance west + SP	320.53			
(2) tick prevalence ~ nest type + nest age + distance south-east + distance west + SP	319.29			
(3) tick prevalence ~ nest type + distance south-east + distance west + SP	318.49			
(4) tick prevalence ~ nest type + distance south-east + SP	317.54			
(5) tick prevalence ~ nest type + SP	316.8	3.73	4	>0.05
(1) tick prevalence ~ nest position+ nest opening + SP	195.64			
(2) tick prevalence ~ nest position+ SP	193.64	2	1	>0.05
(1) tick prevalence ~ temperature mean + temperature SD + moisture soil + moisture nest mat + SP	185.48			
(2) tick prevalence ~ temperature mean + temperature SD + moisture nest mat + SP	183.48			
(3) tick prevalence ~ temperature SD + moisture nest mat + SP	181.49			
(4) tick prevalence ~ moisture nest mat + SP	179.93			
(5) tick prevalence ~ SP	179.42	6.06	4	>0.05

Table S4.4. Model selection based on Akaike Information Criterion (AIC). Numbers in brackets represent the different scenarios derived from the full model (assessed in the study) to reach the best model (in bold). Significant differences between the full and the best model (p-value) according to Chi-square test. Independent variables: nest type (artificial, natural covered and natural open nests), distance to the south-east coast (m), distance to the west coast (m), penguin ectoparasites (abundance), temperature mean (°C) + moisture of nest soil (%) + moisture of nest material (%), SP (sampling seasons: autumn/winter 2016, spring 2016 and autumn/winter 2017) and penguin age (adult penguins and chicks).

Model	AIC	Chisq	df	p-value
y = Penguin body mass				
(1) body mass~ nest type + distance south-east + distance west + penguin ectoparasites + temperature mean + moisture soil + moisture nest mat + SP + age	2175.4			
(2) body mass~ nest type + distance south-east + distance west + penguin ectoparasites + temperature mean + moisture nest mat + SP + age	2173.42			
(3) body mass~ nest type + distance south-east + penguin ectoparasites + temperature mean + moisture nest mat + SP + age	2172.01			
(4) body mass~ nest type + penguin ectoparasites + temperature mean + moisture nest mat+ SP + age	2171.2	4.2	3	>0.05
y = Chick body condition				
(1) condition ~ nest type + distance south-east + distance west + penguin ectoparasites + temperature mean + moisture soil + moisture nest mat + SP	80.49			
(2) condition ~ nest type + distance south-east + distance west + penguin ectoparasites + temperature mean + moisture nest mat + SP	78.54			
(3) condition ~ distance south-east + distance west + penguin ectoparasites + temperature mean + moisture nest mat + SP	76.59			
(4) condition ~ distance south-east + penguin ectoparasites + temperature mean + moisture nest mat + SP	75.57			
(5) condition ~ distance south-east + penguin ectoparasites + temperature mean + SP	74.82			
(6) condition ~ penguin ectoparasites+ temperature mean + SP	74.58	5.91	5	>0.05
y = Penguin haematocrit				
(1) haematocrit ~ nest type + distance south-east + distance west + penguin ectoparasites + temperature mean + moisture soil + moisture nest mat + SP + age	835.44			

(2) haematocrit ~ nest type + distance south-east + distance west + penguin ectoparasites + temperature mean + moisture soil + moisture nest mat + age	832.66			
(3) haematocrit ~ nest type + distance west + penguin ectoparasites + temperature mean + moisture soil + moisture nest mat + age	830.73			
(4) haematocrit ~ nest type + distance west + penguin ectoparasites + temperature mean + moisture nest mat + age	829.05			
(5) haematocrit ~ nest type + distance west + penguin ectoparasites + temperature mean + age	827.34			
(6) haematocrit ~ nest type + distance west + penguin ectoparasites + age	826.19	9.25	5	>0.05
y = Penguin total plasma protein				
(1) total plasma protein ~ nest type + distance south-east + distance west + penguin ectoparasites + temperature mean + moisture soil + moisture nest mat + SP + age	250.48			
(2) total plasma protein ~ nest type + distance south-east + penguin ectoparasites + temperature mean + moisture soil + moisture nest mat + SP + age	248.48			
(3) total plasma protein ~ nest type + penguin ectoparasites + temperature mean + moisture soil + moisture nest mat + SP + age	246.76			
(4) total plasma protein ~ nest type + temperature mean + moisture soil + moisture nest mat + SP + age	245.47			
(5) total plasma protein ~ temperature mean + moisture soil + moisture nest mat + SP + age	244.51			
(6) total plasma protein ~ temperature mean + moisture nest mat + SP + age	243.22			
(7) total plasma protein ~ moisture nest mat + SP + age	243.11	7.37	6	>0.05

Chapter 5

The efficacy of a modified Berlese funnel method for the extraction of ectoparasites and their life stages from the nests of African penguins

(Prepared for *Parasitology Research*)

Abstract

The Berlese funnel method and variations of this method, is commonly used for the extraction of arthropods from various substrates such as soil, leaf litter and nest material. Little is however known about its effectivity in extracting ectoparasites from penguin nests. Using the African penguin as a model, 278 nests at five penguin colonies were sampled along the south-western coast of South Africa in 2016 and 2017. Nest samples were subjected to a modified Berlese funnel system with naphthalene as a repellent. Thereafter all remaining ectoparasites were removed by hand sorting. Compared to total counts, flea (combined life stages and larvae) and total ectoparasite abundance and prevalence were significantly lower with the modified Berlese funnel method. In addition, adult flea and tick prevalence (but not abundance) was significantly lower with the modified Berlese funnel method compared to total counts. Ectoparasite abundance was significantly positively correlated between the two extraction methods. It is evident that the modified Berlese funnel system fails as a quantitative method and can only provide a crude indication of the incidence of ectoparasites in penguin nests.

Key words: Modified Berlese funnel; hand sorting method; ectoparasites; African penguin nests.

Introduction

The soil matrix can sustain a broad diversity of small arthropods, including predatory (e.g. Mesostigmata, Coleoptera, Hymenoptera and Pseudoscorpiones; Ruf 1998; Sotherton 1984; Parisi et al. 2005), fungivorous (e.g. Collembola and Orobatida; Hyvönen and Persson 1996), and parasitic arthropods (e.g. Siphonaptera, Ixodida and Mesostigmata; Clayton and Walther 1997). Studies on soil arthropods often require the use of techniques that provide results that are as close as possible to the existing counts in the sample, however, not all techniques are equally effective (Auerbach and Crossley Jr 1960). Several methods exist to extract the arthropod fauna in soil samples, ranging from mechanical to behavioural methods (Macfadyen 1953; Southwood and Henderson 2000). Mechanical methods include hand sorting, sieving, flotation of microarthropods by immersing the soil in dense salt solutions, elutriation, centrifugation and extraction based on lipophilic properties of the cuticle (Salt and Hollick 1944; Moldenke 1994). The easiest and cheapest mechanical method is hand sorting, i.e. systematically working through each soil sample (Smith et al. 2008). Challenges using this method include that it relies on the knowledge of the person executing the process to recognise and correctly identify the arthropods; it can be difficult to process if the soil has a high proportion of mud and clay and it is time-consuming (Moldenke 1994; Southwood and Henderson 2000; Smith et al. 2008).

Behavioural methods, where the aim is to induce the arthropod to abandon the soil sample, include funnels (dry and wet) that use natural drying by air temperature (e.g. Winkler bags); a source of heat and light such an electric lamp (e.g. Berlese-Tullgren funnels, Kempson bowl extractor) or fumes from a chemical (e.g. naphthalene) as stimuli (Macfadyen 1953; Brown 1973; Southwood and Henderson 2000). Among the behavioural methods, the Berlese-Tullgren funnel is one of the most traditional and widely used methods to extract soil arthropods (André et al. 2002). The initial system was developed by Antonio Berlese, who used heat to drive arthropods out of a sample located in a tray on top of a metal funnel, inducing them to fall into a collecting container (Berlese 1905). Tullgren then improved the apparatus in 1918 using an electric bulb to combine the stimuli of heat and light above the sample, driving arthropods down the funnel away from the desiccation source into a lethal solution (Macfadyen 1953). The Berlese funnel has subsequently gone through many modifications (Table 5.1). For the purpose of this study, we will refer to the Berlese funnels that use dry extraction. The use of Berlese funnels is advantageous because researchers can leave the funnel almost unattended. The method also produces relatively fast results and extraction can be done on a large number of samples simultaneously. Furthermore, these funnels can handle soil samples that contain a

great amount of plant material, be built with low cost materials and function using chemicals, which allow for the device to be transported and used in remote places (Southwood and Henderson 2000; Bano and Roy 2016). However, they also have their limitations. Since its original design did not consider the full quantitative detection of fauna in a soil sample, its extraction efficiency may be too low to record arthropod abundance (Macfadyen 1953). The funnels also do not extract immobile stages (e.g. eggs, pupae) and soil sample conditions (e.g. high water content) can affect the effectiveness of extraction (Barton 1995; Southwood and Henderson 2000). When comparing Berlese funnel methods to other extraction methods the opinions are diverse. Some authors have criticised the method and found that other techniques, such as Winkler extractor (Kalif and Moutinho 2000) or direct counts (Forsslund 1948) are able to collect higher numbers of arthropods from a soil sample. Other authors however, subsequently found that the Berlese funnel is still more efficient in recording arthropods compared to Winkler extractor or hand sorting (Sakchoowong et al. 2007).

Table 5.1. The Berlese funnel method (dry extraction) and some subsequent modifications

Funnel name	Modification	Reference
Berlese funnel	Initial funnel using heat as a repellent	Berlese 1905
Berlese-Tullgren funnel	Uses a light bulb as a heat and light source	Tullgren 1918
Modified Tullgren funnel	Uses a hot wire coils as a heat source	Ford 1937
Modified Tullgren funnel	Introduction of a gap to separate the edges of the sample and the funnel walls to prevent water condensation	Haarløv 1947
Expedition funnel apparatus	Uses paraffin as a source of heat making the funnel more portable	Macfadyen 1953
High gradient funnel	Uses a metal baffle plate that creates a definite gradient of temperature and humidity	Macfadyen 1953
Smaller funnel extractor with air conditioning	Uses heat source from above and provides circulation of cooler air and humidity in the lower surface	Macfadyen 1961
Kempson bowl extractor	Uses a bowl instead of a funnel shape immerse in a cold-water bath, which offers high humidity. It uses a light source that switches on in pulses and gradually.	Kempson et al. 1963
High-efficiency extractor	Soil samples are inserted in inverted aluminium sleeves sealed with cloth over funnels. A space is left between the funnels and the sample bottom. Vial stoppers are set at the end of funnel stems. Light bulbs are inserted in beverage cans supported on top of the funnel. These modifications provide a high gradient of temperature and reduce contamination.	Crossley and Blair 1991

Extraction apparatus	Uses a sample holder that allows access to the sample to keep the friability of the sample.	Barton 1995

The Berlese funnel is still widely used in research on soil microfauna and has frequently assisted the study of arthropods present in bird nests (Murray and Vestjens 1967; Merino and Potti 1996; Rendell and Verbeek 1996; Kim et al. 2017).

The nests of penguin species, such as rockhopper (*Eudyptes chrysocome*), royal (*Eudyptes schlegeli*), Humboldt penguin (*Spheniscus humboldti*), little penguin (*Eudyptula minor*) and Galápagos penguin (*Spheniscus mendiculus*) can harbour numerous nest-dwelling parasitic and predatory arthropods, that include hard and soft ticks (e.g. *Ixodes* spp., *Ornithodoros* spp., respectively), mites (Dermanyssidae, Laelaptidae) and fleas (e.g. *Parapsyllus* spp., *Pagipsylla* spp.) (Murray and Vestjens 1967; Brandão et al. 2014). However, the diversity of arthropods present in African penguin (*Spheniscus demersus*) nests is scarcely known. To date, there is only one study that describes the population structure of the soft tick *Ornithodoros capensis* found in the nests of African penguins at a single locality along the west coast of South Africa (Daturi 1986). This study was conducted using a modified Berlese funnel system where a 400-ml soil sample, collected from each nest, was divided into two portions of 200 ml and placed on a wire mesh (outer holes 1 cm). Naphthalene (100 gm) was positioned above the sample and aluminium foil was used to seal the top of the funnel for 24 hours. In the study the efficiency of the Berlese funnel method was initially confirmed by hand sorting the first 18 samples, and subsequently continued to only use the funnel method, possibly due to indifferences between the sample methods The Berlese funnel system was also previously used to extract arthropods in or around nests from other penguin species such as royal penguins and gentoo penguins (*Pygoscelis papua*) (Murray and Vestjens 1967; Dahl 1970). However, these studies only reported extraction of arthropods with the funnel method.

In the present study, the first aim was to assess the efficacy of a modified Berlese funnel method in extracting ectoparasites from African penguin nests and secondly, establish if the pattern observed is similar for all parasite taxa and different life stages. We predict that the modified Berlese funnel method will only extract a subset of the total parasites in the nests and that the effectiveness of the funnel method will be influenced by the mobility of the taxa and/or life stages.

Materials and methods

Field sample collection

A total of 278 African penguin nests were sampled at five penguin colonies along the south-western coast of South Africa during the autumn/winter season of 2017 (sample size: Dassen Island 40 nests; Dyer Island 40 nests; Robben Island 40 nests; Simon's Town 40 nests; Stony Point 118 nests). A 200-ml sample was collected from the central area of each penguin nest, and included nest material (e.g. seaweed, sticks and stones) and soil (soil and sand from the ground surface to 2 cm depth) The samples were collected using a hand spade and placed in a plastic container (sealed with a plastic screw-top lid) and kept cool until examination (within 24 hrs from collection).

Laboratory analysis

In the laboratory, each nest sample was divided into two equal portions (100 ml each) and placed in a modified Berlese funnel system similar to Daturi (1986). Each portion was placed on a round filter paper which was placed on a wire mesh (holes 2 cm) that was inserted in a plastic funnel (made using a recycled 2-lt soft drink bottle) (Fig. 5.1A). The diameter of the filter paper (ca. 6 cm) was smaller than that of the wire mesh (ca. 10 cm) in order to leave enough space (ca. 2 cm) for the ectoparasites to fall through the mesh holes once they left the sample (Figure 5.1B). A cloth bag (cheesecloth style fabric) with 100 gm of naphthalene mothballs was hung over the sample from a horizontal wooden stick placed in the upper opening of the funnel (Figure 5.1C). The upper section of the funnel was sealed with a plastic cover and secured with an elastic band (Figure 5.1D). A plastic container with 70% ethanol was placed below the funnel to collect the ectoparasites that fell through. The collection container was also placed in a cloth bag, which was tied to the funnel stem with a plastic cable tie to prevent ectoparasites from escaping in the lower area of the funnel (Figure 5.1B). The soil samples were left in the funnel unit for 24 hours.

Figure 5.1. The modified Berlese funnel system used to extract ectoparasites from the nests of African penguins. Soil samples placed on a round filter paper on the wire mesh (A). Lateral view of the funnels with the plastic containers inside a cloth bag attached to the stem of the funnels (B). Naphthalene balls inside a bag hanging above the soil sample (C). Top view of the funnels sealed with a plastic cover (D).

After the extraction time, the collection container was closed with a plastic lid and labeled with the reference code and the method of extraction (i.e. funnel method). Likewise, the soil samples were recovered from each funnel, placed in their original containers, and preserved with 70% ethanol. Subsequently, each soil sample was systematically examined by hand using a stereo microscope (Leica KL300 LED, Wetzlar, Germany). The ectoparasites, collected through hand sorting, were placed in a plastic container with 70% ethanol and labelled with the reference code and method of extraction (i.e. by hand). All ectoparasites were identified to species level, using taxonomic reference keys (Bedford 1934; Jordan 1942; Arthur 1963; Segerman 1995), counted and the life stage recorded.

Statistical analysis

Ectoparasite abundance (i.e. number of ectoparasites out of the total number of nests examined; Bush et al. 1997) and prevalence (i.e. number of infested nests out of the total number of nests examined; Bush et al. 1997) were assessed per extraction method, i.e. modified Berlese funnels and total counts (ectoparasites extracted using the funnel and the subsequent hand sorting). Whenever the life stage was morphologically different (e.g. adult fleas vs. flea larvae), we

analysed ectoparasite burdens per life stage assuming that certain life stages are more mobile compared to others (Bitam et al. 2010). Data were tested for normality using the Shapiro-Wilk test. We used generalized linear models (GLMs) to assess differences between the two extraction methods correcting for colony. Abundance data was first modified (by adding the value of 1 and log-transformed) due to an excess of zeros in the data (highly skewed). We then used zero-inflated regression with a negative binomial residual distribution (zeroinfl function from the 'pscl' R package; Jackman 2017). Parasite prevalence was obtained by transforming count data into presence/absence, and differences between methods were assessed using GLM with a binomial distribution (function glm()). The relationship between the ectoparasites abundance recorded with the two methods was assessed using Spearman correlation tests. Statistical tests were conducted in R 3·4·3 (R Core Team 2017).

Results and Discussion

Fleas (*Parapsyllus humboldti* and *Echidnophaga gallinacea*) and soft ticks (*O. capensis* s. s.) were recorded from the African penguin nests. Since *E. gallinacea* was only found in penguin nests on Dassen Island, we combined the total counts of this flea species with those of *P. humboldti* to obtain the abundance and prevalence of fleas as a group. Ticks (adult, nymphs and larvae) were combined in a single group given that all life stages are mobile. In total 9237 ectoparasites were recorded of which 6905 were fleas (flea larvae 6518 and adult fleas 387) and 2332 were ticks. Mites (Acari) were also present the nest samples. However, because the taxonomic differentiation between parasitic and non-parasitic mite taxa requires experience taxonomic expertise this taxon as a whole was not considered in the analysis.

In general the abundance recorded for fleas (combined life stages and larvae) and total ectoparasites combined were significantly higher in the total counts compared to the modified Berlese funnel method only (z-statistic all $p<0.05$), while there was no significant difference in the abundance of adult fleas and ticks between the two methods (Table 5.2, Fig. 5.2 and 5.3). In addition, the funnel method significantly underestimated the prevalence of total ectoparasite, fleas (adults and larvae) and ticks in penguin nests (Table 5.3 and 5.4). This indicates that the modified Berlese funnel method on its own is not effective in removing all the ectoparasites from nest samples and that a similar (in the case of adult flea and ticks) or larger number (flea larvae and total ectoparasites) of ectoparasites are still in the nest samples after the funnel method (Table 5.2). This is in agreement with previous reports that recorded a higher mean abundance of diverse arthropods in soil (e.g. Oribatida mites, Parasitiformes, Trombidiformes and Collembolan) with hand sorting (direct counts) compared to the Berlese funnel method

(Forsslund 1948; Macfadyen 1953). However, these studies removed two separate samples from the same nest and each sample was subjected to a different method (hand sorting or funnel extraction). This is in contrast to the present study where the same sample was subjected to both methods. The latter may be a more accurate method to assess the efficacy of the modified Berlese funnel method as it is based on the same sample.

The present study further highlights variation in the efficacy of the modified Berlese funnel method between parasitic life stages. Although total counts did record a higher abundance for ticks and fleas (combined life stages), the funnel method was more effective in removing ticks and adult fleas compared to flea larvae. This is evident in the fact that there was no significant difference recorded in the tick and adult flea abundance between the two methods, while a significant difference was recorded for flea larvae (Table 5.2, Fig 5.2). This pattern support previous studies that also recorded variation in the efficacy of the Berlese funnel method between arthropod taxa and life stages (e.g. Macfadyen 1953; Auerbach and Crossley Jr 1960; Sakchoowong et al. 2007; Smith et al. 2008; Barberena-Arias et al. 2012). The premise of behavioural methods, such as the Berlese funnel, in extracting arthropods depends on the mobility and ability of the arthropods to move away from the repellent (Southwood and Henderson 2000). It is therefore inferred that the pattern observed in the present study is due to the limited ability of flea larvae to migrate out of the soil sample. All life stages of soft ticks have well-developed legs and a strong capacity for movement, even migrating between nests in a seabird colony when searching for a suitable host (Sonenshine 1993; Dupraz et al. 2017). Adult fleas are morphologically adapted to move through the substrate of a bird's nest due to their laterally compressed body and head, and their strong legs and joints, which allow them to walk, climb or jump (Krasnov 2008). On the other hand, flea larvae have a worm-like slender body without legs and although they are capable of some mobility through their setae and by contracting and extending their body it is limited (Bacot and Ridewood 1914; Bitam et al. 2010). Variation in the efficacy of naphthalene to act as a repellent may also contribute to the difference observed between flea life stages. Naphthalene seems to be effective for both fleas and ticks (Medleau and Miller Jr 1983; Daturi 1986). However, it is possible that flea larvae are less sensitive and or exposed (“stuck” in the soil material) to the fumes and might not respond equally fast to naphthalene in a 24-hour exposure period (Oliver and Beattie 1996).

Table 5.2. Relationship between ectoparasite extraction method (modified Berlese funnel and total counts (funnel + hand sorting)) and the abundance of ectoparasites in nests of African penguins. Type and family of regression models according to data distribution: zero Inflated negative binomial (ZINB). Significant values: ***=<0.001, **=0.001-0.01, *=0.01-0.05, ·=non-significant.

Dependent variable	Model	Intercept	Independent variable	Estimate	Std. Error	z value	p-value
Adult fleas	ZINB	Funnel	Total count	0.11471	0.06459	1.776	.
		Dassen Island	Dyer Island	-0.22876	0.16394	-1.395	.
		Dassen Island	Robben Island	-0.28298	0.12065	-2.345	*
		Dassen Island	Simon's Town	0.16961	0.0974	1.741	.
		Dassen Island	Stony Point	-0.20505	0.09353	-2.192	*
Flea larvae	ZINB	Funnel	Total count	0.15207	0.05786	2.628	**
		Dassen Island	Dyer Island	0.52895	0.13352	3.961	***
		Dassen Island	Robben Island	0.47934	0.10658	4.498	***
		Dassen Island	Simon's Town	0.62271	0.1044	5.965	***
		Dassen Island	Stony Point	0.4914	0.0885	5.553	***
Total fleas	ZINB	Funnel	Total count	0.16928	0.05551	3.05	**
		Dassen Island	Dyer Island	0.33477	0.12407	2.698	**
		Dassen Island	Robben Island	0.33631	0.10204	3.296	***
		Dassen Island	Simon's Town	0.53327	0.10049	5.306	***
		Dassen Island	Stony Point	0.39031	0.0853	4.576	***
Total ticks	ZINB	Funnel	Total count	0.08793	0.08005	1.098	.
		Dassen Island	Dyer Island	0.08948	0.22003	0.407	.
		Dassen Island	Robben Island	0.05178	0.24283	0.213	.
		Dassen Island	Simon's Town	0.15948	0.18383	0.868	.
		Dassen Island	Stony Point	0.54415	0.16458	3.306	***
Total ectoparasites	ZINB	Funnel	Total count	0.18103	0.05268	3.436	***
		Dassen Island	Dyer Island	0.08524	0.11549	0.738	.
		Dassen Island	Robben Island	0.26848	0.10249	2.62	**
		Dassen Island	Simon's Town	0.42107	0.09937	4.238	***
		Dassen Island	Stony Point	0.38547	0.08487	4.542	***

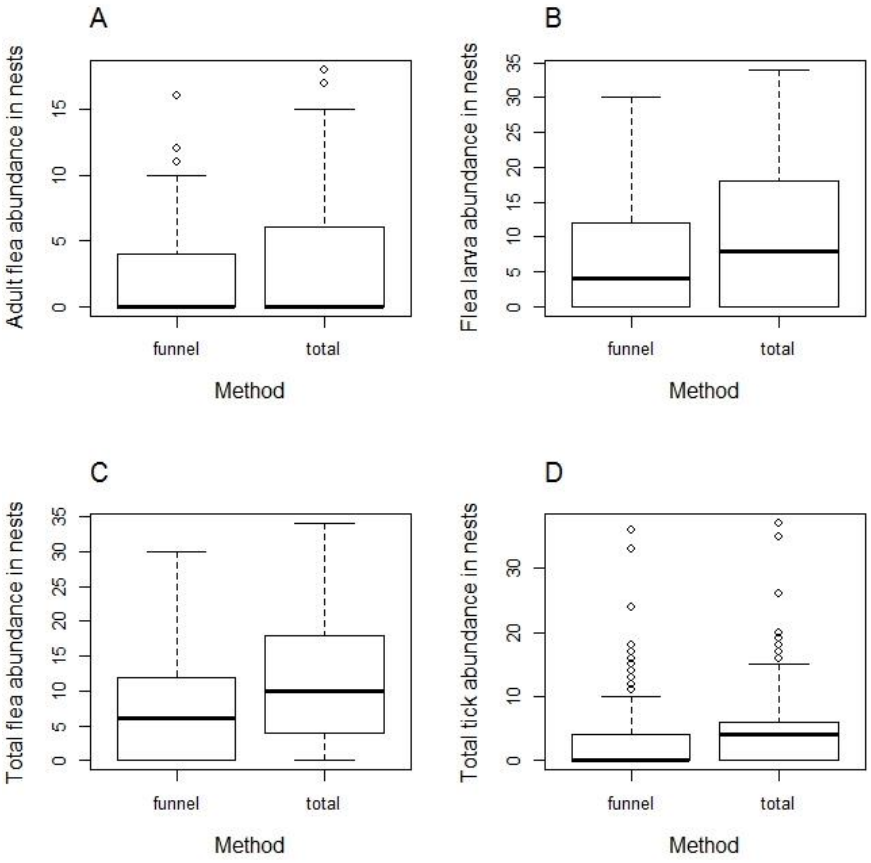

Figure 5.2. Flea and tick abundance (ln(n+1)) per extraction method (modified Berlese funnel and total counts (funnel + hand sorting)) in nests of African penguins. Ectoparasitic groups: Adult flea abundance (A); flea larvae abundance; Total flea (adults and larvae) abundance (C); Total ticks abundance (D).

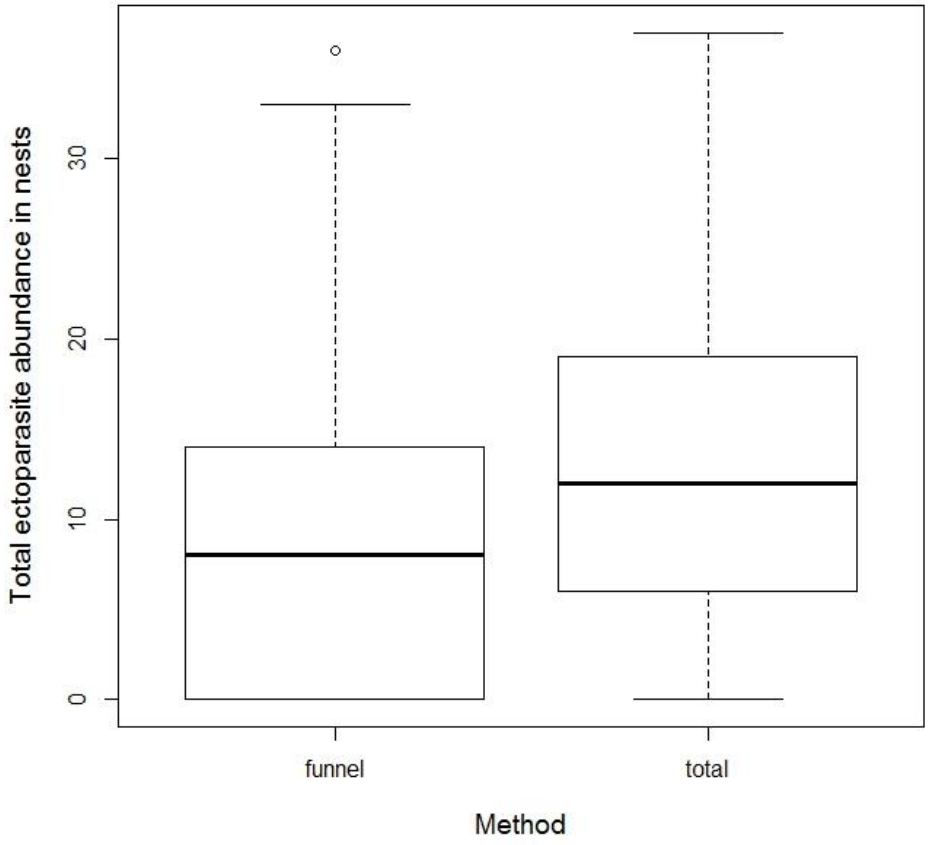

Figure 5.3. Total ectoparasite (fleas and ticks) abundance (ln(n+1)) per extraction method (modified Berlese funnel and total count (funnel + hand sorting)) in nests of African penguins.

Lastly, there was a significant positive correlation in the ectoparasite abundance between the two extraction methods. This was the pattern for adult fleas ($r_{Spearman}$=0.73, p<0.001), flea larvae ($r_{Spearman}$=0.88, p<0.001), total fleas ($r_{Spearman}$=0.89, p<0.001), total ticks (0.76, p<0.001) and total ectoparasites ($r_{Spearman}$=0.89, p<0.001). This suggests that the methods correspond with each other, and that the pattern recorded for the modified Berlese funnel is relatively comparable to the pattern recorded by total counts. The modified Berlese funnel method can thus be used as a qualitative method to compare the occurrence of ectoparasites, especially more mobile taxa, within or between penguin colonies.

Table 5.3. Prevalence (%) of ectoparasites per extraction method (modified Berlese funnel and total count (funnel + hand sorting)) in nests of African penguins.

	Extraction method (%)	
Parasitic group	Funnel	Total count
Adult fleas	25.90	42.09
Flea larvae	56.83	69.78
Total fleas	63.31	75.18
Total ticks	32.37	51.44
Total ectoparasites	74.1	86.33

Table 5.4. Relationship between ectoparasite extraction method (modified Berlese funnel and total count (funnel + hand sorting)) and the prevalence of ectoparasites in nests of African penguins. Type and family of regression models according to data distribution: glm 'binomial'. Significant values: ***=<0.001, **=0.001-0.01, *=0.01-0.05, ·=non-significant.

Dependent variable	Model	Intercept	Independent variable	Estimate	Std. Error	z value	p-value
Adult fleas	glm'binomial'	Funnel	Total count	7.82E-01	1.90E-01	4.123	***
		Dassen Island	Dyer Island	-1.25E+00	4.19E-01	-2.974	**
		Dassen Island	Robben Island	2.56E-15	3.43E-01	0	.
		Dassen Island	Simon's Town	1.02E+00	3.35E-01	3.045	**
		Dassen Island	Stony Point	1.04E-01	2.80E-01	0.372	.
Flea larvae	glm'binomial'	Funnel	Total count	0.613306	0.186688	3.285	**
		Dassen Island	Dyer Island	-1.672348	0.347203	-4.817	***
		Dassen Island	Robben Island	-0.058743	0.342796	-0.171	.
		Dassen Island	Simon's Town	0.121442	0.348638	0.348	.
		Dassen Island	Stony Point	-0.005026	0.281848	-0.018	.
Total fleas	glm'binomial'	Funnel	Total count	0.61414	0.19543	3.143	**
		Dassen Island	Dyer Island	-1.51337	0.34472	-4.39	***
		Dassen Island	Robben Island	0.20054	0.36614	0.548	.
		Dassen Island	Simon's Town	0.27203	0.36976	0.736	.
		Dassen Island	Stony Point	0.06367	0.2935	0.217	.
Total ticks	glm'binomial'	Funnel	Total count	9.64E-01	1.96E-01	4.916	***
		Dassen Island	Dyer Island	-5.66E-15	3.94E-01	0	.
		Dassen Island	Robben Island	-4.39E-01	4.22E-01	-1.038	.
		Dassen Island	Simon's Town	1.54E+00	3.63E-01	4.232	***
		Dassen Island	Stony Point	1.85E+00	3.13E-01	5.92	***

Total ectoparasites	glm'binomial'	Funnel	Total count	0.8786	0.2348	3.742	***
		Dassen Island	Dyer Island	-0.8554	0.3438	-2.488	*
		Dassen Island	Robben Island	0.5134	0.3867	1.328	.
		Dassen Island	Simon's Town	1.2602	0.4543	2.774	**
		Dassen Island	Stony Point	1.065	0.3265	3.262	**

Conclusions

It is evident that the modified Berlese funnel system, with naphthalene as repellent, fails as a quantitative method for ectoparasite extraction from penguin nests. At most the method can provide a crude indication of the occurrence of ectoparasites in nests. Further, intrinsic factors associated with parasite life stages can result in intraspecific variation in the efficacy of the modified Berlese funnel system. The findings of the study are important for future studies on parasite demographics in African penguin nests, as the sole use of the modified Berlese funnel method will severely affect the quality of the data and subsequent deductions thereof.

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Chapter 6

Synthesis and Recommendations

The African penguin (*Spheniscus demersus*) is a charismatic seabird that feeds on marine fishes and breeds on island and mainland colonies along the coast of South Africa and Namibia. This endangered species has experienced a drastic and continuous population decline in recent years. Among available legislation to provide for the African penguin's long-term protection and survival in the wild, the Department of Environmental Affairs developed a biodiversity management plan. Conservation agencies are sensitive to any potential threats that can contribute to a further decline in population numbers. Recent reports of nest and chick abandonment by adult penguins along with the presence of soft ticks on the penguins and around their nests in the Stony Point colony, raised concerns that parasites may pose a new and additional threat to the survival of the species. The current lack of information on the factors that drive parasite infestations on African penguins and in their nests gave impetus to research the *in situ* factors that shape parasite populations (ecto-, haemo-, and helminth parasites) at different scales (regional and local) and assess the impact of the current parasite infestation levels on the general health of African penguins.

Chapter 2 sets out to determine the factors that shape the diversity and infestation levels (richness, abundance and prevalence) of parasite taxa on African penguins and in their nests at a regional scale. The study was conducted at five colonies (three island and two mainland) along the west and south coast of South Africa. From the data, it is clear that parasite infestations vary between colonies with the eastern mainland colonies recording higher ecto-, haemo-, and helminth parasite infestations compared to the islands and more western colonies. In particular, Stony Point was identified as having the highest tick infestations. Further, there were parasite-specific responses to the factors that shape infestations with ectoparasite infestations and haemoparasite incidence directly and indirectly influenced by nest density, respectively. On the other hand, helminth infestations may be driven by the distribution of helminth-infected prey fish and the exploitation of rock lobsters (which feed on snails that act as first intermediate hosts of fish helminths) with a higher incidence of helminth infestations in penguins at the two eastern mainland colonies. It is possible that the eastward migration of fish stocks may also indirectly facilitate ectoparasite and pathogen distribution as the three eastern colonies (Simon's Town, Stony Point and Dyer Island) recorded higher total nest density compared to the western colonies, which in turn would facilitate higher parasite loads. In general, penguin chicks were especially susceptible to parasite infestations, possibly due to their more immature immune system and their close association with the nest during the first 80 days of their life. The regional study provides preliminary data on a positive relationship

between warm dry seasons and ectoparasite infestations and haemoparasite incidence. However, given the short duration of the study, the validity of this relationship will need to be tested in future studies.

After establishing that penguins are commonly infected by hematophagous parasites, **in chapter 3** the study sets out to record general health parameters for wild African penguins and establish if the current level of parasite infestation adversely affects the general health of penguin populations**.** Several standard clinical parameters (body mass, body condition of chicks, haematocrit and total plasma protein) were recorded from adult penguins and chicks at the five African penguin colonies. Clinical parameters varied among colonies; however, penguins in the most western colony (Dassen Island) recorded a lower body condition (chicks) and TTP (chicks and adults), while penguins in the two eastern mainland colonies (Simon's Town and Stony Point) recorded lower haematocrit values. Ecto- and haemoparasite species richness negatively affected clinical parameters whereas helminth parasites positively affected it. Particularly in the Stony Point colony, tick abundance also exerted a negative effect on clinical parameters. In addition, in Stony Point the body condition of chicks was lower in spring compared to autumn/winter and season was a significant predictor in all cases. The study was limited in the number of years and seasonal data at multiple colonies. Therefore, results on the seasonal effect are limited to a local scale (Stony Point). Although the negative effect of parasite richness on the general health status of penguins is evident, it is likely that stress factors, such as increase in temperature during spring as well as variations in the availability of prey fish at each particular colony can exacerbate the effect of parasites. Based on the current results it is clear that penguins are capable of maintaining healthy conditions in the presence of low parasite infestations. However, when penguins are challenged with stressful conditions, such as high nest density, higher parasite infestations, extreme climatic conditions and poor food resources (the latter indicated by a lower body condition in chicks), then it does appear that their health is adversely affected.

Following on from the regional study, **chapter 4** focuses on the effect of nest characteristics, microclimate and spatial position, on the ectoparasite burdens in penguin nests and evaluates the subsequent effect on penguin health at the Stony Point penguin colony. Microclimatic conditions in artificial nests and nests located closer to the coast were associated with higher ectoparasite abundance and prevalence. Warmer and drier nests promoted ectoparasite infestations, an observation which supports the positive relationship between warm dry seasons

and ectoparasite and haemoparasite incidence seen in the regional study. The microclimate in more open nests was less suitable for ectoparasite development and seem to negatively affect the body mass of penguins. These results suggest that, although artificial and natural covered nests protect penguins from extreme climatic conditions and facilitate breeding success, their use should be considered with care given that they can facilitate higher ectoparasite burdens and vector-borne diseases. This study provides a better understanding of the importance of local-scale factors on parasite populations and distribution. It is advisable that data on nest ectoparasite burdens and microclimatic conditions must be recorded for artificial nests in other colonies. Only then can recommendations be made on the role of artificial nests in facilitating ectoparasite infestations.

The Berlese funnel system, or modifications thereof, have been used in previous studies on ectoparasite demographics in penguin nests. **Chapter 5** examines the efficiency of a modified Berlese funnel (with naphthalene as repellent) as a quantitative method to extract soft ticks and fleas from African penguin nests. The study uses nest material samples collected from the five African penguin colonies. For all parasite taxa and life stage the modified Berlese funnel system underestimated parasite abundance and prevalence. The comparative study also revealed that the funnel method was more effective in extracting mobile life stages (adult fleas) compared to less mobile stages (flea larvae). The findings of this study are relevant for future investigations on microarthropods present in penguin nests. It is advisable to use a combination of both methods (modified Berlese funnel and hand sorting) to obtain a quantitative estimation of ectoparasite abundance and prevalence. Although the funnel method does have limitations it reduces the time spent on hand sorting.

Recommendations from the study:

- Active long-term and seasonal monitoring of parasite infestations in penguin nests and the general health parameters of penguins in colonies that have a high nest density. This will identify colonies that are at risk and confirm the importance of warm and dry seasons for parasite infestations and penguin health.
- Include breeding success data recorded per nest type to assess how the different nest types and associated parasite loads impact breeding success and chick mortality. This data will better direct management actions.

- Investigate available options to increase the colony size of Stony Point, thereby lowering the overall nest density. This may help to reduce parasite loads, and associated parasite impact on penguin health during periods of high stress (e.g. extreme climate and limited food availability).
- Improve surveillance of disturbances in the colonies (e.g. predators and humans) and declare marine protected areas around all penguin colonies. The former may reduce stress factors and facilitate a stronger immune response in penguins, while both may facilitate a healthy ecosystem where high rock lobster populations will maintain lower snail populations and thus reduce the incidence of helminth parasites in prey fish. Marine protected areas will also increase local fish stocks by countering overfishing.
- Extend the study to African penguin colonies located along the east coast of South Africa in order to assess if the factors that shape parasite infestations and penguin health along the south-western coast are also relevant under different environmental conditions (climatic and food availability).
- Identify potential sites for the establishment of new colonies using habitat suitability models. This may reduce the feeding pressure on limited food resources and facilitate an increase in the number of breeding pairs.